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Determining the Transport Enhancement of Sodium Fluorescein in Mechanically-Loaded Canine Tibia

Andrew Joseph Zak
Cleveland State University

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DETERMINING THE TRANSPORT ENHANCEMENT OF SODIUM FLUORESCEIN IN MECHANICALLY-LOADED CANINE TIBIA

ANDREW J. ZAK

Bachelor of Chemical Engineering

Cleveland State University

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We hereby approve this thesis for

Andrew Joseph Zak

Candidate for the Master of Science in Chemical Engineering degree for the

Department of Chemical and Biomedical Engineering

and the CLEVELAND STATE UNIVERSITY

College of Graduate Studies

Thesis Chairperson, Dr. Joanne Belovich

Department & Date

Thesis Committee Member, Dr. Caroline Androjna

Department & Date

Thesis Committee Member, Dr. Ronald Midura

Department & Date

Thesis Committee Member, Dr. Surendra Tewari

Department & Date

Student’s Date of Defense: 8/27/2015
DEDICATION

This thesis is dedicated to my parents, Terry and Donna Zak. Your unending love and support have always carried me throughout all my academic endeavors.
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DETERMINING THE TRANSPORT ENHANCEMENT OF SODIUM FLUORESCEIN IN MECHANICALLY-LOADED CANINE TIBIA

ANDREW J. ZAK

ABSTRACT

Quantitative research concerning the impact of mechanical loading on the transport properties of bone has several critical applications. One such application is the effect of a microgravity environment, where the lack of mechanical forces on bone has been shown to negatively impact both growth and repair. A method has been developed in our lab that can potentially allow for the measurement of the effective permeability of large molecules (i.e., 300-15,000 Da in size) in bone tissue under both unloaded and mechanically loaded conditions. In proof-of-concept experiments, previous students have measured the effective diffusivity of the model solute, sodium fluorescein (376 Da) in a sample of unloaded bone tissue. A mechanical loading system has been modified to measure the effective permeability of sodium fluorescein for a bone beam undergoing four point bend testing in a bioreactor system in order to quantify the effect of mechanical loading on solute transport. The first goal of the present work was to validate that deflection of the bone beam was occurring at applied displacements of less than 40 μm. Once the deflection of the bone beam in the mechanical loading system was validated, the primary objective of measuring the transport parameter for sodium fluorescein in canine cortical bone under unloaded and loaded conditions could be
achieved. The average value and standard error of this parameter for loaded samples was determined to be $3.70\times10^{-8}\pm1.31\times10^{-8}$ cm$^2$s$^{-1}$ (n=5), and $6.59\times10^{-9}\pm2.46\times10^{-9}$ cm$^2$s$^{-1}$ (n=4) for unloaded samples. Although a student’s t-test showed that the loaded and unloaded values were not statistically different (p=0.08), this is likely due to the small number of samples. These preliminary results do show that the transport parameter of sodium fluorescein in cortical bone increased by more than factor of 5 with the addition of mechanical loading.
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1.1. Importance of Understanding Solute Transport in Bone

The ability to study and quantify transport properties such as the diffusion coefficient in compact bone is of great importance to the field of medicine and biomedical engineering. A wide range of future applications may be heavily based in molecular transport, especially with new drug delivery methods that are performed on the nano scale. Diseases such as osteoporosis, or the loss of bone density, result in more than 8.9 million fractures worldwide each year\textsuperscript{13}. The 1.5 million fractures caused by osteoporosis in the United States each year lead to more than half a million hospitalizations, over 800,000 emergency room visits, and more than 2.6 million physician office visits, driving the total direct care expenditures on osteoporotic fractures to over $12 billion dollars per year\textsuperscript{5}. Better treatment of bone-related diseases requires a fundamental understanding of drug and solute transport at the macro and molecular levels.
Additionally, ongoing research in the area of space flight continues to examine the effects of microgravity on bone loss and the probability of bone fractures \(^8\). Bone-loading exercises are an established protocol for prevention of bone density loss in the microgravity environment. To date however, the role of molecular transport of key growth factors and signaling molecules in the maintenance of bone is not fully understood\(^1\),\(^6\). Furthermore, the effects of mechanical loading on solute transport and ultimately bone growth and/or repair are only recently being investigated and quantified\(^4\),\(^2\),\(^4\).

### 1.2. Problem Statement

The overall purpose of this research is to show whether or not there is a significant enhancement of solute transport in canine tibia when mechanical loading is applied. A procedure has been previously developed to measure the diffusion coefficient or transport parameter of sodium fluorescein (376 Da) in canine cortical bone tissue at the mm-level using fluorescent imaging. This scale was chosen as opposed to individual lacuna-canaliculi systems within the bone matrix since these systems of canals and void space only make up about 3-5\% volume of the cortical bone region. Therefore, studying solute transport across the entire bone tissue may provide more meaningful data for describing the behavior of solute transport in bone. Bone beams were sealed such that only the medial face (most interior of the bone) was exposed to the solute. This setup allowed for the 1-dimensional diffusion of sodium fluorescein to be quantified in the radial direction of the canine tibia. After the bone beam was immersed in the solution for 24 hours slices from the bone beam were imaged. A Matlab script was run to calculate
the effective diffusion coefficient based on the best fit of the diffusion model to the measured concentration profile.

Using the method described above, the effects of mechanical loading on solute transport in bone were studied by incorporating four-point loading to the sample while it was immersed in the same sodium fluorescein solution. Although the present setup adds a convective form of transport, using the Matlab script and diffusion model to calculate a single transport parameter even with mechanical loading allows for a direct quantitative comparison between solute transport in loaded and unloaded bone. The increase or decrease in this transport parameter for the loaded case compared to the unloaded control samples may be interpreted as either an enhancement or reduction of the transport rate.

1.3. Specific Aims

The present research aimed to accomplish the following:

1. **Validate the mechanical loading system by visually capturing the center deflection of the bone beam during four-point load testing.** A high resolution CCD camera was positioned in front of the open port in the 6 well-plate that contained the bone beam on the sample holder. Images were taken after 5 μm displacements had been applied incrementally up to 30 μm. Measurements of the center deflection were made by comparing images using ImageJ software before and after applied displacements.

2. **Determine the Young’s modulus of the bone beams using the mechanical loading system.** A pressure indicating sensor film was implemented to experimentally measure the pressure/force range on the bone beam during mechanical loading. A
small sample of the film was placed between the bone beam and the plunger tips. When a displacement was applied, the pressure from the plunger pushing down against the film and bone beam resulted in a color profile on the film. Samples were sent out to the company to read the pressure profile and return the pressure readings for each film sample. The estimates of these readings, the dimensions of the particular bone beam, and the applied deflection were used to calculate the modulus for the bone.

3. *Show whether or not applying mechanical loading provides enhancement of solute transport in bone, and quantify the degree of enhancement using the current model.* A sample immersion technique and Matlab script had already been developed to obtain concentration profiles of bone beams that had been exposed to sodium fluorescein solutions of various concentrations (0.3-300 μm$^3$). This technique was slightly modified to fit within the mechanical loading system. Encapsulated bone beams were immersed in a 30 μm sodium fluorescein solution for a period of 24 hours before slices of the beam were cut and imaged. This concentration was chosen to ensure that a measurable fluorescence signal could be detected in samples during imaging. Some of these bone samples remained unloaded, while others underwent continuous mechanical loading in the form of a sine wave with amplitude of 18 μm and a frequency of 2.5 Hz. Concentration profiles were generated and transport parameters were determined to compare the unloaded and loaded samples.
1.4. Significance of Work

Successful completion of this research will provide quantitative evidence of the effect of applied loading on the transport of a small molecule solute in cortical bone. If enhancement of solute transport is observed with loading, then this work may shed light on the possibility of other load-induced mechanisms for solute transport through bone. Moreover, measuring the effects of loading on solute transport may provide a more quantitative understanding of mechanotransduction, which has already been shown to play a crucial role in bone maintenance and repair\textsuperscript{12}. Finally, the use of mechanical loading may make its way into drug delivery methods in a further effort to increase the bioavailability of drugs in specific areas.
2.1. Bone Anatomy and Physiology

Among the types of bone present in the human body are long bones such as the tibia, which are characterized by a structure that has a longer length than width. Together the tibia and fibula connect the ankle to the knee in the lower section of the leg and work together to provide stability. Cortical or compact bone, a dense connective matrix, makes up the outermost region of the tibia and gives the bone its tensile and compressive strength to support most of the human body weight. Cancellous or trabecular bone, a porous or “spongy” matrix, is present in the centermost region of the bone and contains the bone marrow with important nutrients and minerals. Figure 2.1 shows the basic anatomical features of a long bone.
The outer surface of the cortical region of the tibia is covered by the periosteum, which is composed of two layers. An outer fibrous layer of dense irregular connective tissue protects the bone from surrounding structures, secures blood vessels and nerves to the surface of the bone, and serves as an attachment site for ligaments and tendons. The inner cellular layer includes osteoprogenitor cells, osteoblasts, and osteoclasts which are intricately involved in the growth and maintenance of the bone. All internal surfaces of the bone are covered by an endosteum layer, which also contains the three types of cells mentioned above.

The matrix of bone connective tissue is made up of both organic and inorganic components. The organic component is osteoid, which is comprised of roughly 90% Type I collagen in addition to various proteoglycans and glycoproteins. These organic components, which make up a total of 1/3 of the bone mass, give bone tensile strength by resisting stretching and twisting, and contribute to its overall flexibility. The inorganic
portion of the bone matrix is made up of hydroxyapatite crystals, which is a calcium phosphate derivative with the formula Ca$_{10}$(PO$_4$)$_6$(OH)$_2$. These crystals harden the matrix and account for the rigidity or relative inflexibility of bone that provide its compressional strength. Additionally, they may serve as buffer source to prevent large fluctuations in serum pH$^{17}$.

Most of the tibia is compact or cortical bone, which is composed of small cylindrical structures called osteons, or Haversian systems, in canines, humans, and other larger mammals. An osteon is the basic functional and structural unit of the compact bone. Osteons are oriented parallel to the diaphysis, or long axial segment of the bone (see Figure 2.1). An example of an osteon microstructure is shown below in Figure 2.2.

Figure 2.2: Osteon and associated microstructures of compact bone$^{17}$. 
Located in the center of the osteon is the Haversian canal through which blood vessels and nerves are located. The surrounding structure of the osteon is formed by concentric lamellae that give the bone strength through an alternating pattern of collagen fibers. In between adjacent concentric lamellae are osteocyte cells which are responsible for maintaining the bone matrix. Osteocytes are found in the small spaces known as lacunae. Each lacuna is connected to the central canal through a series of Canaliculi channels, which allow for nutrients, minerals, gases and wastes to be transported. Finally, Volkmann canals resemble the Haversian canals in that they contain blood vessels and nerves, but run perpendicular to the length of the bone. These allow for multiple osteons to be interconnected and allow for further molecular transport to occur.  

In the present research canine tibia were selected as the choice of animal model due to the structural similarities of compact bone to that in humans. Although much research has been performed on the transport of various solutes in the bones of rats, this model lacks the organization of cortical bone into osteon groups. Moreover, it is important to consider that the thickness of the cortical region of the tibia and other bones may be significantly different depending on the size and weight of the animal species. While solute transport in individual canaliculi in the rat may be analogous to that in humans, transport across the entire tissue may not be comparable due to the structural differences mentioned above.

2.2. Solute Transport: Diffusion and Convection

Solute transport through the bone microstructure has been briefly described above, and a careful explanation of this phenomenon is considered here. Transport in the
present application refers to the mass transfer of solute particles from an area of high concentration (surrounding solution) to an area of low concentration (the bone itself, which has no solute present initially). It is important to distinguish between the two mechanisms of mass transport that take place in cortical bone tissue: diffusive mass transport and convective mass transport. Diffusive mass transport may be defined as the free movement of solute particles from an area of high to low concentration\(^3\). This type of transport may be observed when a piece of bone is simply placed in a solution, as was the case in the sample immersion experiments used to measure effective diffusivity in canine bone tissue\(^\text{10}\). The difference in the chemical potential between the fluid inside the bone and the solution outside the bone, usually approximated by the difference in solute concentration, is the only driving force present to allow solute particles to diffuse into the static fluid within the bone.

Convective mass transport involves the directed movement of solute particles in a moving fluid due to an exterior driving force, such as a pressure gradient or similar pumping activity\(^3\). Diffusive molecular transport may still be present, but the solute particles are further transported due to the bulk flow of the fluid. In the present research, the internal pressure gradients created by the deformation of the bone beam during loading will result in convective transport of sodium fluorescein. This effect will represent an enhancement to the transport of the solute when compared to simple diffusion mass transfer.
2.3. Selection of the Solute

Sodium fluorescein (376 Da) was chosen as the solute in these and previous experiments due to its fluorescent properties and structural similarities to molecules found in bone tissue. The structure of sodium fluorescein is shown below in Figure 2.3.

![Figure 2.3: Structure of sodium fluorescein (376 Da)](image)

This chromophore has well-defined excitation (491 nm) and emission (515 nm) wavelengths, making it a good candidate for use in fluorescence microscopy and spectroscopy. Furthermore, sodium fluorescein is structurally similar to several molecules commonly found in bone tissue, which are shown in Figures 2.4-2.5. Each of these molecules plays an important role in the growth, maintenance, or repair of bone. Vitamin D is responsible for maintaining a balance of calcium and increasing bone remodeling and resorption\textsuperscript{16}. Estrogen and testosterone are both signaling molecules involved in regulating bone resorption\textsuperscript{16}. Thus, measuring the transport parameters of sodium fluorescein may provide an understanding of how other molecules of similar size and structure might behave as well.
2.4. Background on Solute Transport and Effects of Mechanical Loading

Some of the first experiments studying the effects of mechanical loading on solute transport in bone were performed by Knothe Tate et al.\textsuperscript{14,15}. They developed theoretical, \textit{ex vivo}, \textit{in vitro}, and \textit{in vivo} methods to investigate fluid flow and transport of tracer molecules under mechanical loading conditions. The \textit{ex vivo} model was used to study fluid displacements where loading could be well-controlled. An adult sheep forelimb was explanted distal to the elbow joint, and two Schanz screws were inserted through the distal and proximal metaphyses of the metacarpus. Just prior to mechanical loading, one of three solutes (disulphine blue, procion red, or microperoxidase) were injected intraarterially. Using an Instron testing machine, the metacarpus was loaded cyclically
via the Schanz screws with mixed compressive and bending modes for times of 2, 4, 8, and 16 minutes. Strain gauges were used to measure the strain magnitude (0.2% strain) on the anterior side of the mid-diaphysis of the metacarpus at a frequency of 0.5 Hz. Tracer was also injected on the contralateral control side, which was not subject to mechanical lading. Thin sections from the mid-diaphysis were analyzed via light, electron, and confocal microscopy to track the tracer movement and dynamics of load-induced fluid flow.

In the in vitro model, cylindrical specimens were cut from the cortical bone of the adult sheep metacarpus\textsuperscript{15}. Two specimens were placed in a 0.1% procion red solution, one cyclically loaded on the Instron machine while the other served as an unloaded control. The purpose was to determine the relationships between loading parameters (i.e. cycle number, load magnitude, and loading rate) and the extent of deformation-induced fluid displacement. Standard histologic procedures were used to slice and image thin sections at the conclusion of the experiment using fluorescent imaging techniques.

Finally, an in vivo diffusion study was performed in order to investigate the role of tracer molecular weight on the transport rate in bone. These were initially performed without mechanical loading in order to serve as a baseline. Knothe Tate et al. used a four point bending method developed by Akhter et al. on the right rat tibia immediately after anesthesia was given\textsuperscript{14}. The left tibia served as an unloaded control. Twelve rats were injected with tracer simultaneously and divided into four groups. Three groups received 36 cycles of a 65 N load, with frequencies of 0.2 Hz, 2 Hz, and 5 Hz serving as the varying parameter, while the fourth group underwent compressive loading transverse to the long axis of the tibia. Light and transmission electron microscopy techniques were
used to image the sections taken from the explanted tibiae at the conclusion of each experiment.

For the ex vivo experiments, it was reported that the concentration of tracer measured in the mid-diaphysis was significantly higher in the loaded bone compared to the control region. However, no quantitative data was provided to support these claims. The authors mentioned that mechanical loading parameters (i.e. cycle number and loading rate) also had a significant impact on the tracer concentration, but again, made no mention of the final concentration or any other measured quantity. Similar trends were reported for the in vitro and in vivo studies, although diffusion profiles and other data for the in vitro and in vivo models were not included. In another study published by Knothe Tate et al. the in vivo experiment described above was more fully investigated, but no calculation of a diffusion coefficient or similar transport parameter appeared in these results either\textsuperscript{15}. It appears that the analysis of the data collected is greatly lacking in these preliminary studies. It is interesting to note however that the bones of the adult sheep used in the ex vivo and in vitro experiments do show secondary osteon development after the age of 1, which would make this a better model than the rat model used in the in vivo experiments if comparison were to be made to expected transport trends in human bone.

Wang et al. employed a fluorescence recovery after photobleaching (FRAP) technique in order to measure the transport of sodium fluorescein in the bone lacunar-canalicular system\textsuperscript{25}. Here a diffusion coefficient for sodium fluorescein in an unloaded mouse tibia was measured as a baseline for future experiments. In this application of the FRAP technique, 0.2 mL of sodium fluorescein (10 mg/mL) was injected into the tail vein of mice, and allowed to circulate for 20 min. The left tibia of each animal was
exposed, and a microscope was focused 30-50 μm below the periosteum layer, which was left intact. High intensity laser irradiation was applied for 15 seconds, providing enough energy to effectively photobleach or “eliminate” the fluorescence of the injected tracer in a particular lacuna and adjoining canaliculi. Images were taken over the course of 120 seconds after photobleaching until a plateau of fluorescence was reached as the non-photobleached tracer began to diffuse into the canaliculi and lacuna. They then calculated a diffusion coefficient based on the intensity profile of the FRAP images using a source-sink model for the lacunar-canalicular system. The neighboring lacuna that had not been photobleached served as the source for sodium fluorescein, while the lacuna that underwent high intensity irradiation was treated as a sink in this model. An average diffusion coefficient of \(3.3 \pm 0.6 \times 10^{-6} \text{ cm}^2/\text{s}\) was calculated, which is on the same order of magnitude as the diffusion of sodium fluorescein in water. This is surprising since the present system is focusing on a single lacuna-canalicular system. The path of a solute molecule would certainly be impeded by molecules and fibers within the canals, which should result in a smaller diffusion coefficient compared to the solute in water.

There are several suspect assumptions and major limitations of the analysis of FRAP data to calculate diffusivities. In the experimental setup, Wang et al. claimed that solute transport occurred via 22% of the surrounding canaliculi, which represents those that had not been photobleached\(^{25}\). However, this figure may be in question due to the fact that the model drawn to illustrate this assumption is only 2-D. It is unclear whether or not the high intensity laser irradiation would have photobleached the canaliculi extending downward in the z-direction below the body of the lacuna. More clarity is needed on how the 22% was arrived at in order to show whether or not this number is
correct, since the 2-D model of the lacuna-canalicular system does not appear to be an accurate representation. No mention was made of the measured concentration of sodium fluorescein at any point in time in the source or in the lacunar-canalicular system, which presents another limitation of this work. Results from our lab indicate that the rates of transport and diffusion coefficients are significantly affected by the solute concentration in the source\textsuperscript{10}, which indicates that the diffusion coefficients calculated by Wang et al. have little meaning. In general, there are many physiological parameters used in the model to calculate the diffusivity, and the sensitivity of the calculated diffusivity to errors in these parameter estimates was not explored.

As mentioned earlier, it is important to consider the animal model when trying to compare diffusion coefficients and other calculated transport parameters to what one might expect in humans. Wang et al. used a mouse model, which lacks the osteon structures observed in human cortical bone. Additionally, the diffusion coefficients measured in prior work from our lab are determined based on the transport of the solute through the entire tissue, not simply in a single lacunar-canalicular system.

Little quantitative data has been published on the effect of mechanical loading on solute transport, particularly in bone. Price et al. (same group as Wang et al.) did however incorporate loading to the experiment described above several years later\textsuperscript{19}. In these experiments mice tibiae were harvested and placed in a mechanical loading system immediately following the sodium fluorescein injection, as shown in Figure 2.6.
Cyclic compression with a 3 N peak load (400 με) was applied at a period of 0.5 Hz. A 4 second rest window was inserted for images to be taken. The same FRAP technique described earlier was utilized in order to measure the real-time intensity of the lacunar-canalicular system under study after photobleaching had occurred. In this case a three compartment model of one photobleached lacuna (sink) and two surrounding lacunae (reservoirs) was used for the simulation as a theoretical basis for calculating the diffusion coefficients.

The experimental data were fit to an empirical, 2 parameter model of exponential form. The transport rate, $k$ and recovery time constant, $\tau$ were calculated from both unloaded and loaded experimental data. The loaded transport rate was determined by the slope of the natural log of the normalized intensity ratio vs. time. An average transport rate of 0.024 s$^{-1}$ was determined for the loaded case compared to 0.017 s$^{-1}$ for the unloaded samples. Thus, the overall transport enhancement (ratio of the transport rates)
was reported as 1.31 ± 0.24. The time constant τ at which 63% of the fluorescence intensity had been recovered in the photobleached lacuna was 43 seconds for the loaded bone and 65 seconds without loading present. The fluid velocity in the canaliculi was also calculated from the data and the model, demonstrating that convection does occur during loading\textsuperscript{19}.

There were several significant limitations with the analysis of the results obtained in this study. A diffusion-convection equation was presented early on in the paper, but not used in any of the calculations. The transport rate and time constant were empirical parameters that had no direct relationship to this equation. Therefore, the connection to the theory and equation that includes a convective term to the actual results reported appears to be missing. Although this equation may have been used to obtain results from the simulation, no mention was made of the baseline value for the unloaded cases. It would have been helpful to see whether or not the simulation predicted the values that were reported for unloaded cases from this group’s research published in 2005 that was described earlier. As was the case with their previous experimental design, it should be noted that a mouse model was again used, which makes a comparison to the transport rates that one might expect in human bone questionable at best.

Arkill et al. studied the effect of static load on the transport of sodium fluorescein and rhodamine B in the deep and calcified zones of articular cartilage and subchondral bone of mature horses\textsuperscript{2}. An intact perfused limb was mounted to a loading rig, and a pin running through the shaft of the third metacarpal parallel to the long axis of the bone was loaded to 1500 N by a hydraulic arm. The limb was exposed to a solution of sodium fluorescein and rhodamine B (0.1 mg/mL) in PBS for a period of 1.5 hours. The
contralateral limb served as the unloaded control. Several 20 μm thick transverse sections were cut from the center of the plug containing cartilage, the calcified zone, and a thin layer of bone. These sections were then prepared and imaged using a 5X objective under epi-illumination (fluorescent filtering). Fluorescence intensity profiles were taken and corrections to background autofluorescence were made by imaging samples that had not been previously exposed to the sodium fluorescein or rhodamine B solutions.

It was found that the addition of mechanical loading appeared to have no effect on the uptake of rhodamine B. However, it is interesting to note that the relative concentration of sodium fluorescein in the calcified zone and cortical bone region decreased by a factor of three compared to the unloaded control. The authors claim that this was an expected result for a charged solute, citing the possibility of tissue consolidation or compression of the subchondral vasculature due to electrostatic exclusion. It is important to consider that while these phenomena may explain these results, there are several major limitations with the experimental design of this experiment. First of all, a static load was applied for a period of 1.5 hours, which might represent the horse standing in a fixed position for that period of time. The transport mechanism may certainly be different if cyclic loading were applied, which would model the horse walking or running. The constant change in forces experienced under cyclic loading would create a much different pressure gradient than static loading. Additionally, it is important to recognize that the cartilage regions studied are much different in mechanical and chemical properties compared to cortical bone. The similarities in imaging methods make for an interesting comparison to our group’s present research, which is described in the next section.
2.5. Transport in Unloaded Canine Tissue

An *in vitro* method has been developed in our lab to measure the diffusion coefficient of sodium fluorescein in the radial direction of canine tibia bone tissue\textsuperscript{10}. Beams of approximately 3 mm x 3 mm x 20 mm were cut from 4-5 different sections of the tibia. These bone beams were sealed with dental resin on all sides except for the medial (innermost) face of the bone beam. In this way, radial diffusion from the inner to outmost part of the bone could be measured. The encapsulated bone beam was placed in a 50 mL solution of sodium fluorescein. Concentrations ranging from 0.3-300 μM were used in order to determine the effect of solute concentration on the rate of transport. The beams were removed from the solution after a period of 24 hours, and 100 μm thick slices were cut and mounted with VectaShield mounting medium. Samples were immediately imaged using fluorescent microscopy techniques, revealing an intensity profile with a high concentration at the exposed end and a low concentration at the sealed end. A Matlab code was written to convert the intensity profile of each image to a concentration profile and find the best-fit diffusion coefficient from the model equation.

Measurements of the diffusion coefficient of sodium fluorescein in cortical bone using this technique were confirmed using a standard two-chamber diffusion system. As the solute concentration increased, the diffusion coefficient decreased, ranging from $1.6 \times 10^{-7} \pm 3.2 \times 10^{-8} \text{ cm}^2 \text{s}^{-1}$ at 0.3 μM to $1.4 \times 10^{-8} \pm 1.9 \times 10^{-9} \text{ cm}^2 \text{s}^{-1}$ at 300 μM. The results show that there is no significant difference in mean diffusion coefficient obtained using the two measurement techniques on the same sample, $2.0 \times 10^{-8} \pm 4.7 \times 10^{-9} \text{ cm}^2 \text{s}^{-1}$ (sample immersion), compared to $3.3 \times 10^{-8} \pm 6.6 \times 10^{-9} \text{ cm}^2 \text{s}^{-1}$ (diffusion chamber)\textsuperscript{10}. 
Wang et al. reported diffusion coefficients that were two orders of magnitude larger than the ones reported from our lab ($10^{-6}$ cm$^2$s$^{-1}$ vs $10^{-8}$ cm$^2$s$^{-1}$)$^{25,10}$. The values reported from our lab are much smaller since our system studies diffusion in the radial direction through the entire cortical bone tissue, much of which is made up of a dense matrix with very little porosity. Therefore, it is to be expected that the diffusion coefficient measured through the cortical bone matrix would be much smaller than the diffusion coefficient measured through an individual lacuna-canaliculi system, which is the primary source of the porosity in cortical bone. It should also be noted that the diffusion coefficients calculated by Wang et. al. are on the high end of values calculated for similarly sized molecules, and actually approach the diffusion coefficient of sodium fluorescein in water$^{25}$. The order of magnitude of the diffusion coefficient obtained using the sample immersion method in our lab ($10^{-8}$ cm$^2$s$^{-1}$) fall in the middle of reported values for diffusion coefficients of sodium fluorescein and molecules of similar size (300-3000 Da) in cortical bone ($10^{-6}$-$10^{-10}$ cm$^2$s$^{-1}$)$^{9}$.

2.6. Loading Methodologies and Present Work

The present research focuses on utilizing the sample immersion technique described above with the addition of mechanical loading in order to quantify the convective enhancement of solute transport in a bone model similar to that of humans. While certain mechanical loading systems that were presented earlier in this section applied compressive loading to the entire bone from the proximal end$^{19}$, other mechanical loading devices such as the Instron machine were utilized to apply both compressive and bending loads to both small sections of the bone and the entire limb itself$^{14}$. 
Shimko et al. designed a mechanical loading device for natural and engineered scaffolds and tissues that utilized a 4 point loading setup\textsuperscript{21}. A tissue sample was placed on a sample holder directly under a plunger apparatus. A piezoelectric transducer was placed above the plunger in order to apply loading to the sample. Our mechanical loading system is very similar to the design presented here with only a few modifications. A 4 point loading setup was preferred to 3 point loading since the 4 point loading setup provides a larger region of constant strain compared to the 3 point setup, which results in a large peak stress at the point of contact where loading is applied. The four point bend test gives a better representation of the kind of compression and tension forces present on a bone under physiological conditions while walking or standing.

Although several methods were presented citing the effect of mechanical loading on solute transport, many of these methods were shown to have significant limitations. In some cases, the diffusion coefficient or other transport parameters were simply unable to be measured\textsuperscript{14}. Other groups calculated a diffusion coefficient and cited an enhancement of solute transport for only a single lacuna-canaliculi system, which focuses on a very small and unrepresentative part of the larger bone matrix\textsuperscript{19}. Our present work will seek to quantify the enhancement of solute transport through the entire bone matrix utilizing a 4 point loading system in order to provide data on a more physiologically relevant scale.
CHAPTER III
MATERIALS AND METHODS

3.1. General Processing of Canine Tibiae

3.1.1. Preliminary Dissections

Hind limbs were obtained from a 30 kg, 1 year old canine in 2006 (Lot Number: 06D325) by previous students at the Cleveland Clinic Lerner Research Institute following IACUC guidelines and regulations. Muscle tissue was removed from the tibia and femur, which were then separated just below the patella. After removing the fibula with a scalpel, the outermost surface of the tibia was cleaned using gauze pads and phosphate-buffered saline (PBS, 1% sodium azide) to remove any remaining fascia. The ends of each tibia were cut and labeled near the patella (proximal) and talus (distal) regions, leaving 4-5 inches of the long bone as shown in Figure 3.1.
Tibiae were stored in 50 mL conical vials (Fisher Scientific) in a PBS solution at -4°C in the walk-in refrigerated storage room at the Lerner Research Institute. Each vial was labeled to indicate left or right tibia and included the year, species (canine), and lot number of the animal as well as the researcher name and date of processing.

3.1.2. **Further Processing into Bone Beams**

Each tibia was then divided into 4-5 sections depending on the length of the tibia. Prior to sectioning, bone marrow was removed by flushing PBS from a syringe into the medullary cavity and wiping away any excess marrow with gauze pads. A Labcut 1010 diamond blade saw (Extec Corp., Enfield, CT) was then used in to further process the tibia into individual sections as shown in Figure 3.2.
5-10 bone beams with approximate dimensions of 20 mm x 3 mm x 2 mm were cut from each section using the diamond blade saw as shown in Figure 3.2. The height of each bone beam was measured along its length using a set of calipers (Mykita). If the bone beams were found to be uneven, sequentially finer grit sandpaper was used to make the beams more uniform. The most proximal end on the periosteum layer of each beam was marked with a biocompatible marker (Viscot Medical, NJ). Bone beams were stored in 50 mL conical vials at -4°C in a fresh PBS solution and labeled as before with the specific section number to track what part of the tibia each beam came from.

3.2. Mechanical Loading System Validation Procedures

3.2.1. Mechanical Loading System Assembly

The basic assembly of the mechanical loading system is briefly described here, with a detailed description provided in Appendix A. This system is derived from a design by Shimko et al.\textsuperscript{21} shown in Figure 3.3. Tissue scaffolds and other “beam-like” materials were placed in the Teflon tissue holders (Figure 3.3E) which rested in the six-well plate between the base plate (Figure 3.3-4) and the sterility plate (Figure 3.3-2).
piezo transducers (Figure 3.3A) could be controlled to apply a certain displacement to the tissue plunger (Figure 3.3B), which is in direct contact with the scaffold or beam. An o-ring was inserted in between the tissue plunger and the sterility plate in order to prevent the two metal parts from grinding against each other.

Several modifications were made to the mechanical loading system designed by Shimko et al. in order to meet the needs of the current experimental design. Piezo transducers (Physik Instrumente (PI), Germany) with a height of approximately 3’’ were used in place of the original transducers due to different displacement capabilities. Metal shims and screws of appropriate heights were thus selected to connect the top two metal plates together with the transducers in between. Additionally, since the manufacturer of the original o-rings no longer produced that product with the same 25 durometer rating, silicone rubber o-rings with a 40 durometer rating were substituted (Scientific Instrument
The six-well tissue plastic culture plate was replaced with one that was made from a Pexiglas® G acrylic sheet (Altuglas, Philadelphia, PA). This was done since the sterility plate located directly above the six-well plate was slightly modified to fit a rounded well plate. Moreover, the solid acrylic plate appeared to be less likely to move and deform during mechanical loading experiments compared to its plastic counterpart. The assembled system is shown in Figures 3.4a and 3.4b.

In the modified system, a bone beam was placed on the sample holder inside one of the wells of an acrylic 6-well plate as shown in Figure 3.5a. For this particular well, the 3/16” hole was expanded to 5/16” to provide a better view of the bone, as shown in Figure 3.5b.
A stainless steel circular stabilizing piece shown in Figure 3.6a was placed directly on top of the sample for a two-fold purpose: to prevent the sample from moving within the holder and to keep the plunger piece aligned on top of the bone beam. The opening of the stabilizing piece allowed the plunger tips to come in direct contact with the beam, as shown in Figure 3.6b.

The sterility plate, o-rings, and tissue plunger were positioned above the sample holder and well plate as shown in Figure 3.7.
In order to accommodate bone beams of different heights, plastic shims made in-house were placed around the screws above the sterility plate as shown in Figure 3.8. The shims provided enough clearance so that the transducers were just in contact with the plunger without providing too much of a pre-load to the bone beam.

The transducer assembly was put together separately from the bottom portion of the mechanical loading system. Screw inserts were inserted into the stationary end of the
transducer as shown in Figure 3.9a. These inserts could be adjusted so that the body of the transducer remained stable and stationary between the two metal plates as shown in Figure 3.9b.

Figure 3.9a-b: Screw insert for piezo transducer (a) and transducer assembly (b).

The plunger could be pushed downward by controlling the mobile component of the piezo transducer that was positioned directly above and in contact with the plunger, as shown in Figure 3.10.

Figures 3.10: Four-point load testing setup.
The piezo actuators were connected to the E-516 Controller Unit (PI) as shown in Figures 3.11a and 3.11b. LabVIEW software provided by PI for the E-516 Controller Unit was used to apply certain displacements to the transducer and consequently the plunger and bone beam. The protocol and instructions for using the E-516 Controller Unit are included in Appendix B.

3.2.2. Sample Preparation and Imaging

The center of the bone beam was marked with a thin vertical line of black waterproof eyeliner gel as a reference for positioning the beam in front of the camera. Additionally, small dots (approximately 10-50 μm diameter) of the same gel were applied at various locations near the center of the beam as points to be followed as the beam was being deflected. Using the E-516 Controller Unit and LabVIEW software, displacements of 0-30 μm were applied with 5 μm increments to the bone beam.

After each displacement was made, an image was taken using the ImperX camera system (ImperX; Boca Raton, FL). The charged-coupled device (CCD) camera was set up on an adjustable boom stand (Diagnostic Instruments, Inc. Sterling Heights, MI) as

Figures 3.11a and 3.11b: Mechanical loading system and E-516 Controller Unit.
shown in Figure 3.12a so that the camera lens could be positioned directly in the line of view of the bone beam. An ACE illuminator (B&B Microscopes, LTD. Warrendale, PA) was used to provide additional lighting so that bone beam was visible to the camera lens as shown in Figure 3.12a.

Figures 3.12a-b: Camera setup on boom stand (a) with additional lighting applied to view bone beam (b).

640x480 resolution images of the bone beam (Figure 3.13) were taken of the bone before and after applied displacements using predefined settings (10 bit, 30 fps, 33 ms exposure time) in StreamPix (NorPix Inc., Montreal) digital video recording software as shown in Figures 3.14a and 3.14b.

Figure 3.13: Horizontal center of bone beam marked with black gel eyeliner.
Figures 3.14a-b: Horizontal center of bone beam marked with black gel eyeliner at displacements of 0 μm (a) and 30 μm (b); Horizontal yellow line drawn across images to show vertical displacement

Since images were recorded manually, the frame rate was only applicable for capturing video of the bone beam when a continuous sine wave loading was applied. These images were exported from StreamPix as JPEG files and opened in ImageJ (National Institute of Health; Bethesda, MD), a Java-based image processing program. A bitmap analysis was performed using ImageJ, creating a matrix of the intensity value of each pixel over the entire image. This matrix was exported into a Microsoft Excel spreadsheet. Detailed instructions on setting up the ImperX camera and using StreamPix are provided in Appendix C.

3.2.3. Analysis of Images

Two different methods were employed for analyzing images and quantifying the vertical deflection of the horizontal center of the bone beam. The first method employed a bitmap analysis in order to measure the change in intensity between the bone and the background in order to measure the deflection. The change in intensity between each successive row was calculated in Microsoft Excel, and the position of the maximum
change in intensity was found for each column. A visual inspection of Figures 3.14a and 3.14b indicates that the largest change in intensity should occur at the boundary between the bone (high intensity) and the background (low intensity). In order to calibrate a scale between the distance in pixels and micrometers for each image, an image of a microscale slide was taken at the same magnification as the images of the bone beam. Therefore, by tracking how far (in pixels) the boundary layer moved from image to image after a certain displacement was applied, the physical deflection of the bone beam was quantified.

The second method for measuring the center deflection used merged images of the bone beam taken before and after applied displacements. An image taken without any applied displacement was combined with an image taken at displacements between 0-30 μm in ImageJ. Figure 3.15a shows the bone beam at 0 μm displacement on the green channel, while Figure 3.15b shows the bone beam at 30 μm applied displacement on the red channel.

Figures 3.15a-b: Horizontal center of bone beam at 0 μm displacement on green color channel (a) and 30 μm displacement on red color channel (b).
Since the images were merged on two different color channels, the deflection of the bone beam could be quantified by measuring the thickness of the red region in the merged image shown in Figure 3.16. This red region represents the overlay of the bone beam from the image with an applied displacement, and therefore does indeed show the actual deflection of the bone beam.

![Figure 3.16](image-url)

Figure 3.16: Composite image of Figures 3.15a and 3.15b merged together. Yellow lines point to the red overlap region, which represents the vertical deflection at 30 μm applied displacement.

In order to measure the deflection of the bone beam from the merged images, the relationship between the pixels and physical distance was first established. The picture of the micron scale that was used for the first method was opened in ImageJ. A line representing a distance of 1000 μm was drawn as shown in Figure 3.17. Thus the physical distance was calibrated to the number of pixels on that line, and the pixel to micron ratio (0.337 pixels/micron) was determined and set as a global variable. Therefore, whenever line measurements were made, the physical distance of the line in microns could be easily measured.
Approximately 40 evenly-spaced vertical lines were drawn along the 1.9 mm horizontal length of the red overlap region in Figure 3.16. Measurements of the lines, which represent the vertical deflection at that particular horizontal position, were exported into Microsoft Excel. An average vertical deflection and standard deviation for the length of bone in view was determined at each applied displacement.

### 3.2.4. Pressure Indicating Sensor Film Application

In addition to measuring the deflection of the bone beam, measurements of the pressure and forces on the beam were desired in order to obtain more accurate estimates of the modulus. Since the piezo actuators in the mechanical loading system were not designed to provide accurate force measurements, a pressure indicating sensor film (Sensor Products, Inc., Madison, NJ) was utilized. A thin strip (15 mm x 4 mm) of Medium Range (1400-7100 psi) pressure indicating sensor film was cut using 28 mm blade diameter rotary cutters (Fiskars, Helsinki, Finland) on a rotary cutter mat as shown in Figure 3.18. The film was inserted between the bone beam and the plunger tips as
shown in Figure 3.19. A complete procedure for cutting and using the sensor film is included in Appendix E.

Figures 3.18 and 3.19: Cutting strips of pressure indicating sensor film (3.18) to be placed on bone beam (3.19) to measure pressure under mechanical loading.

Once the mechanical loading system was reassembled with the pressure indicating sensor film, a displacement of 40 μm was applied to the bone beam. The maximum allowable displacement was applied to ensure that enough pressure would be applied to the film to leave a visible, detectable mark. When enough pressure was applied, a colorimetric reaction caused the film to turn magenta at the point of pressure as shown by the sample strips Figure 3.20. S1-S7 represents 7 samples utilized for the same bone beam before (S1-S3) and after (S4-S7) the bone beam was sanded down to make the beam more even. Based on the intensity of the magenta markings, the pressure could be determined from a predefined scale. Samples were sent to the manufacturer (Sensor Products Inc.) to be scanned and analyzed for pressure readings.
Once the pressure gradients and readings were obtained, estimates of the force on the bone at the “points” in contact with the plunger tips were calculated, along with the modulus of elasticity of the bone beam. For a beam supported on both end with two equal loads as shown in Figure 3.21, the maximum vertical deflection, $y$ at the center is given by:\(^\text{18}\):

$$y = \frac{Wa}{24EI} \left(3l^2 - 4a^2\right) \quad (1)$$

In Equation 1, $y$ is the vertical displacement [m], $W$ is the load at each point [N], $a$ is the distance between the applied load and nearest support [m], $E$ is the modulus of elasticity...
[Nm²], I is the moment of inertia [m⁴], and l is the length of the beam between the two bottom supports [m]. The moment of inertia, I for a beam is given by¹⁸:

\[ I = \frac{1}{12}wh^3 \]  \hspace{1cm} (2)

In Equation 2, w is the width of the bone beam [m] and h is the height of the bone beam [m]. Since the vertical deflection was already known (40 μm), and the load was determined from the pressure film readings, the modulus of elasticity could be calculated.

3.3. Sample Immersion Procedures

3.3.1. Encapsulating Bone Beams

After validation of the four-point load testing, bone beams were encapsulated using a silicone sealant (Locktite). A syringe needle (Medline, Mundelein, IL) was used to apply a thin layer of sealant to all faces of the bone beam except the bottom surface, which was left unsealed as shown in Figure 3.22. This bottom face was on the innermost part of the bone section as shown in Figure 3.2 such that diffusion of the solute could be measured in the radial direction of the bone.
Figure 3.22: Encapsulating the bone beams in silicone sealant for sample immersion experiments.

The sealant was allowed to cure and dry for a period of at least 48 hours before any excess silicone was removed using a scalpel (Fisher Scientific). Small incisions were made into the sealant such that the plunger tips would still be in direct contact with the bone, but minimal to no liquid could enter from the top surface of the bone that was otherwise completely sealed. Sealed bone beams were then stored in 50 mL of PBS (1% sodium azide) at -4°C until further use in sample immersion experiments.

### 3.3.2. Sample Immersion Experiments (Unloaded and Loaded)

The validation procedure described in Sections 3.2.1 and 3.2.2 was again used to ensure that the bone beam was still being displaced after the sealing process. Once the displacement of the bone was again visually confirmed, the port was plugged with a 5/16” fine thread stainless steel screw. Approximately 3 mL of a 30 μM sodium fluorescein solution was dispensed into the well using a syringe. The volume of solution was chosen such that the bottom surface of the bone beam was completely immersed in the solution as shown in Figure 3.23.
The mechanical loading system was then put back together and moved to an incubator (Fisher Scientific) at 37°C, 5% CO₂ in air as shown in Figure 3.24. The wire leads from the transducers were fed through a port in the back of the incubator (Figure 3.25) and connected to the E-516 controller unit, which was placed on top of the incubator. A complete setup of the mechanical loading system in the incubator is shown in Appendix D.

Figures 3.24 and 3.25: Mechanical loading system setup in incubator.
For an unloaded trial, the procedure described above was used without any use of the E-516 Controller Unit such that no displacement was applied to the bone beams. The bone beam was simply immersed in the solution for a period of 24 hours. For the loaded trials, a sine wave with amplitude of 18 μm (maximum displacement of 36 μm) and a frequency of 2.5 Hz was applied continuously for the 24 hour period. The amplitude and frequency chosen correspond to loading conditions that would be experienced during a fast walking pace. Continuous loading was chosen for the entire 24 hours since it was desired to see if the maximum loading conditions would show any significant difference in solute transport compared to the unloaded case.

3.3.3. Post Processing and Imaging

After the 24 hour immersion period, the bone beam was rinsed with a PBS solution (1% sodium azide) and the silicone sealant was removed. Using the diamond saw, at least four 100 μm slices were cut from the region of the bone beam between the two plunger points as shown in Figures 3.26 and 3.27. Slices were only used from this section to ensure that all of the images would be taken of the part of the bone beam undergoing the same strain at any given point in time.
Once slices were obtained, calipers (Mykita) were used in order to verify that the slices were of uniform thickness. Uneven slices were sanded down and rinsed with a PBS solution. Samples were then mounted to glass slides with two small drops of VectaShield mounting media for imaging.

Slices were imaged using a Leica DM4000B microscope (Leica Microsystems; Mannheim, Germany) with a FITC fluorescence filter. The microscope was fitted with a QImaging Retiga 2000R CCD camera with Image Pro Plus 7.0 software. The mercury lamp bulb was turned on and allowed to warm up for at least 20 minutes prior to imaging. Once slides were loaded the bone slice was brought into focus using the joystick controller to adjust the x, y, and z dimensions accordingly. The microscope settings were adjusted to 10X magnification, 2x2 binning, a gain of 8 and exposure time of 25 ms for taking images. For the purposes of this work, different gains and exposure times were also investigated for the best image quality and model analysis. Prior to scanning, 15-20 predictive autofocus points were obtained by scanning certain regions of the bone slice and refocusing the image at each of those points. This allowed for a better image of the bone slice if the z dimension topography was uneven in certain places. Once these
predictive autofocus points were saved, 4 x 4 and 5 x 7 images of the bone sample and background were taken. Additionally, an image was taken of a bone sample that had not been exposed to sodium fluorescein as a control to determine the background autofluorescence.

3.3.4. Data Analysis

A bitmap analysis was performed in Image Pro Plus, creating a matrix of intensity values for the entire 2400 x 3600 image. Every 10th point in each row and column was collected, thus reducing the matrix to 240 x 360. Previous work had shown that there was no significant difference in the intensity profile after down sampling had occurred. Moreover, down sampling also decreased the computation time to run the Matlab script. The matrix of intensity values was exported into a Microsoft Excel spreadsheet.

Data analysis was performed using MATLAB custom-written code, which is included in Appendix G. First, the autofluorescence value had been determined by measuring the average intensity of the “blank” bone sample that had not been exposed to sodium fluorescein. This value was set as a parameter in the Matlab Code, and was subtracted from the intensities in all the sample immersion specimens. The average intensity was calculated for each column at each unit of distance (0.012 mm) from the exposed endosteal edge of the imaged bone sample (Figure 3.28).
To generate the normalized concentration profile from the intensity profile, the following equations were used. The conservation of mass of the solute (fluorescein), assuming no reaction and one-dimensional transport by diffusion only, using Fick’s Law of Diffusion, is described by\(^{10}\):

\[
\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial y^2} \tag{3}
\]

with the following initial and boundary conditions:

\[
0 \leq y \leq L, \quad t \geq 0, \quad C = C_o
\]

\[
y = 0, \quad t \geq 0, \quad C = C_s
\]

\[
y = L, \quad t \geq 0, \quad \frac{\partial C}{\partial y} = 0 \tag{4}
\]

where \(C\) is the concentration of the solute (fluorescein) in the tissue, \(C_s\) is the solute concentration at the surface exposed to the solution, and \(C_o\) is the initial solute concentration in the tissue, with all concentrations assumed to be proportional to fluorescence intensity. The coordinate \(y\) is the distance from the exposed face, \(L\) is the
total length of sample, \( t \) is time, and \( D \) is the effective diffusion coefficient of the solute in the tissue.

The solution to the one-dimensional diffusion equation, in finite medium, is given by\(^\text{10} \):

\[
\theta = 1 - 2 \sum_{n=0}^{\infty} \left( \frac{(-1)^n}{(n+\frac{1}{2})\pi} \cos \left( n + \frac{1}{2} \right) \pi \eta \right) \exp \left[ -(n + \frac{1}{2})^2 \pi^2 \tau \right] \tag{5}
\]

The dimensionless variables are defined as:

\[
\theta = \frac{C - C_o}{C_s - C_o}, \quad \eta = \frac{y}{L}, \quad \tau = \frac{tD}{L^2} \tag{6}
\]

\( C_o \) was set to 0 since the autofluorescence value was previously subtracted from the measured intensities. The value of \( C_s \) was calculated from the average of the first five intensities. The intensity profile obtained for each sample was fit to Eq. 5 by minimizing the sum of the squares of the error (SSE) in order to determine the value of the single parameter, the effective diffusivity. Figure 3.29 shows a plot of the concentration profile for the bone sample in 3.28.
3.4. Future Work: Raloxifene Applicability Study

Additional experimental studies were performed in order to assess the potential application of Raloxifene (Sigma-Aldrich) as a fluorescent solute in future applications. The main purpose of these studies was to show whether or not Raloxifene had any measurable fluorescence that could be consistently quantified. A 10 mM stock solution of Raloxifene was prepared by dissolving 4.74 mg of Raloxifene in 1 mL of dimethyl sulfoxide (DMSO). The stock solution was stored in 0.1 mL aliquots at -20°C in polypropylene microfuge tubes (Fisher Scientific) until further use.

Once the samples were allowed to thaw, 0.9 mL of 1% PBS was pipetted to each 0.1 mL aliquot to obtain 1mL of a 1 mM Raloxifene solution. The sample was transferred to a cuvette (Fisher Scientific) and gently stirred before analysis using a fluorescent spectrophotometer (Hitachi). Protocol for use of the Hitchai-7000 spectrophotometer is included in Appendix F. An excitation spectrum and optimum wavelength were first obtained for each sample. Once the excitation wavelength was
defined, an emission spectrum based on the excitation wavelength was produced.
Samples of smaller Raloxifene concentration (0.5 mM) and pH levels (10) were also explored due to issues with solubility and the lack of consistent results for the spectra respectively.
CHAPTER IV

RESULTS

4.1 Mechanical Loading Displacement Validation

To validate the four point bend testing method, bone beam samples were placed in the mechanical loading system. The primary objective for the validation was to show that the applied displacement was observable, measurable, and reproducible. This was especially challenging since the piezo actuators have a maximum displacement of only 40 μm. It was also important to ensure that all components of the mechanical loading system remained stable during testing so that the images taken would show only movement of the bone beam and not any other part of the system (i.e. the well plate).

Several adjustments were made to the original design of the mechanical loading system as detailed in Section 3.2.1. A brief summary of these modifications and validation procedure is presented here. Screw inserts were made for the piezo actuators so that they could be positioned flush against the top plate and would remain stable when displacements were being applied. Additionally, shims (≈50-100 μm thickness) were used to raise the middle plate directly above the plungers. These shims allowed for bone beams of different heights to fit securely in the system. On the new acrylic 6 well plate...
that was made, one of the port diameters was expanded to 5/16” such that the sample holder and bone beam could be viewed by the ImperX camera system. Once these adjustments were made to the system, a vertical band of gel eyeliner was applied to the center of the bone beam as a reference point for taking images. At this point bone beams were left unsealed so that better quality images could be taken without any light scattering from the silicone sealant. Additional lighting was directed through the port so that the camera could pick up an image of the front face of the bone. After 5 μm increment displacements were applied up to 35 μm, an image was taken of the displaced bone beam.

Figure 4.1 shows an example of the images that were taken of the bone beam. The bottom of the front face of the bone beam is seen at the upper region of the image, with the black background located directly below. The vertical black line of gel eyeliner can be seen marking the center of the bone. A few black dots were placed near the horizontal center of the bone beam for additional points of reference and measurement. The total length of the visible region of the bone beam shown in Figure 4.1 is approximately 1.9 mm.

Figure 4.1: Image of the horizontal center of the bone beam marked with gel eyeliner.
A qualitative analysis was first performed by visually comparing images before and after displacement was applied. Image A of Figure 4.2 shows the bone beam without an applied displacement. Image B of Figure 4.2 shows the same bone beam after an applied displacement of 30 μm. The yellow line drawn across the two images in alignment is used to better show the displacement of the bone beam that can be observed in Image B. Image C of Figure 4.2 is the same as Image B, but positioned directly beneath Image A. Images A and C were arranged to show that upon vertical displacement of the bone beam, the horizontal position of the beam remains the same. This observation serves as evidence that the bone beam was stable while displacements were being applied. This visual analysis was repeated once at a 30 μm displacement for the same bone beam to ensure consistency from one applied loading to the next. Moreover, this analysis was also performed and repeated for all applied displacements between 0-30 μm in 5 μm increments. A summary of these findings may be found in the Electronic Appendix.
Figure 4.2A-C: Center of bone beam at 0 μm applied displacement (A) and 30 μm applied displacement (B) horizontally aligned to show vertical deflection. Figure C is the same image as shown in Figure B (30 μm applied displacement), positioned beneath Image A to show no horizontal movement.

Additionally, images were taken of the acrylic well plate while displacements were being applied to the bone beam. Part of the reason for this was that the previously used well plate had become warped on the bottom surface. When displacements were applied with this old plate in use, the entire well plate itself would move. Therefore, the apparent deflection of the bone beam was actually the movement of the entire well plate and sample holder system. As a result, displacement of the bone beam was not actually occurring. Figure 4.3 shows a section of the front face of the well plate when displacements of 0 μm (A) and 30 μm (B) were applied.
Figure 4.3A-B: Front face of well plate at 0 \( \mu m \) applied displacement (A) and 30 \( \mu m \) applied displacement (B) horizontally aligned to show no movement of well plate during loading. The yellow line blue circle around the white mark on the well plate in Figures 4.3A and 4.3B is used for further visual evidence that the well plate remained stationary.

Once the new well plate was inserted, almost no measureable (< 1 \( \mu m \)) vertical movement of the well plate was observed during testing. This analysis confirms that the deflection of the bone beam observed in Figure 4.2 was in fact an actual displacement to the bone beam and not the entire reference frame moving with the bone beam.

Two different analytical methods were used to quantify the center deflection of the bone beam for a given displacement by the piezo actuator. The first method involved combining images before and after displacements were applied and measuring the overlap distance of the two images. This was performed by merging the color channels of the two images together using ImageJ. Figure 4.4 shows the images of the bone beam before (Image A: 0 \( \mu m \) applied displacement, green channel) and after (Image B: 30 \( \mu m \) applied displacement, red channel) displacement was applied. Figure 4.5 shows the two images merged together to form a single composite image. The two blue arrows point to the red band, which represents the actual deflection of the bone beam.
Figures 4.4A-B: Horizontal center of bone beam at 0 μm displacement on green color channel (A) and 30 μm displacement on red color channel (B).

Figure 4.5: Composite image of Figures 4.4A and 4.4B merged together. Blue lines point to the red overlap region, which represents the vertical deflection at 30 μm applied displacement.

A vertical line was manually drawn along red band in Figure 4.5, with the length indicated by the blue arrows, and the length of the line in pixels was measured and converted to microns using ImageJ. The length of the line is considered to be the physical distance of the bone beam vertical deflection at that point. A total of 40 lines were drawn across the 1.9 mm length of the red band in Figure 4.5. This horizontal length represents almost 1/3 of the length of bone beam located between the two plunger
tips (6.05 mm), and 1/10 of the entire length of the bone beam (19 mm). Plots of the measured deflection as a function of the horizontal length measured from the center of the bone beam are shown below in Figure 4.6 for all images with displacements from 5-30 μm.

![Figure 4.6: Measured deflection vs. horizontal bone length from the center of the bone for applied displacements of 5 μm (A), 10 μm (B), 15 μm (C), 20 μm (D), 25 μm (E), and 30 μm (F) using Method 1. The red line in each plot represents the applied displacement.](image)

Although deflections up to 40 μm were possible, it was recommended to operate below the maximum capacity of the actuators to avoid any damage. From the plots in Figures 4.6A-F, the measured deflection appears to be randomly distributed across the
horizontal length near the center of the bone beam without significant variance.

Therefore each data set was averaged to obtain a single center deflection for each applied displacement. Figure 4.7 shows a plot of the average vertical deflection of the bone beam on the y-axis vs. the applied piezo actuator displacement on the x-axis with the standard error shown for each data point.

![Figure 4.7: Average measured deflection of bone beam over 1.9 mm visible region vs. applied displacement of actuator using Method 1. (Mean values shown with SD, n=40). The red line has a slope of unity and represents the theoretical vertical deflection at the center assuming ideal non-deformity of the beam.](image)

Linear regression analysis was performed using the LINEST function in Microsoft Excel with the intercept set equal to 0 for the equation, since an applied displacement of 0 μm would result in exactly a 0 μm displacement. Thus, the linear equation that represents this data is $y = [1.06 \pm 0.02]x$. The slope obtained using this method has a 6% difference from the theoretical slope of unity, assuming that the beam behaves ideally with
deformation during applied displacement. This demonstrates that the applied
displacement of the actuator and the resulting deflection of the bone beam are nearly
equal.

The second method for measuring the bone beam deflection utilizes the difference
in intensity between the bottom edge of the bone beam and the background. A bitmap
analysis of Figure 4.1 reveals that the edge of the bone beam has a high intensity
(≈32,000 intensity units) while the background just below this edge sharply drops in
intensity (≈29,000 intensity units). Therefore, the largest change in intensity should
occur at this boundary layer between the bone and background. The location of this
maximum derivative, as measured from the top of the image, was converted to a distance
in microns using the same pixel to micron ratio that was determined using ImageJ earlier.
The difference in this distance between the image at 0 displacement and at applied
displacement was calculated and considered to be the calculated deflection for the same
bone beam used with the first method.

Plots of the calculated deflection as a function of the horizontal length measured
from the center of the bone beam for this second method are shown below in Figure 4.8
for all images with displacements from 5-30 μm.
Figure 4.8: Calculated deflection vs. horizontal bone length from the center of the bone for applied displacements of 5 μm (A), 10 μm (B), 15 μm (C), 20 μm (D), 25 μm (E), and 30 μm (F) using Method 2. The red line in each plot represents the applied displacement.

One will notice that there is a much greater variability in the deflection measurements along the length of the visible region when using the second method. Although most of the points do lie close to the actual applied displacement, there are certain outliers and even negative deflections that are recorded for all of the applied displacements. Because the bone was marked with a black gel eyeliner along the center, it appears that there are points where the largest change in intensity is no longer at the boundary layer, but at the locations that go from the black gel eyeliner back to the white face of the bone. These
points resulted in much different calculated displacements and even negative
displacements that are shown in Figures 4.8A-F.

Figure 4.9 shows a similar plot of average center deflection of the visible region
of the bone beam vs. the applied actuator displacement. Once again a linear regression
analysis was performed to find the equation that best fit the line drawn for this data set.
Thus, the linear equation that represents this data is $y = [0.93 \pm 0.01]x \, \mu m$. The slope
obtained from the second method has a 7% difference from a slope of unity shown in
Figure 4.9.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure49.png}
\caption{Average calculated deflection of bone beam over 1.9 mm visible region vs. applied
displacement of actuator using Method 2. (Mean values shown with SD, n=640). The red line has a slope of
unity and represents the theoretical vertical deflection at the center.}
\end{figure}

Overall, this second method has a much smaller computational and human time
compared to the first method, but also has a much greater uncertainty, as demonstrated by
the larger standard deviation in Figure 4.9 compared to Figure 4.7. For future studies and
experiments I recommend that the first method of merging the two images together be
used to calculate the center deflection of the bone beam under applied loading based on the better precision.

4.2 Pressure Indicating Sensor Film Application

A pressure indicating sensor film was placed on the surface of the bone in the loading system in order to measure the pressure on the bone beam during mechanical loading. These measurements would allow for better estimates of mechanical properties for the bone beams, including Young’s modulus. Figure 4.10 shows several samples of the film (≈18 mm x 5 mm) after a displacement of 40 μm had been applied. One can see the magenta marks on the film from the plunger tips pushing down on the film and bone.

![Figure 4.10: Pressure indicating sensor film after 40 μm displacement was applied to the bone beam. Samples S1-S3 are samples from a bone beam that was cut but not evenly sanded. Samples S4-S7 are samples from the same bone beam after sanding to even out the top and bottom surfaces.](image)

A qualitative inspection of these samples reveals the usefulness of the pressure indicating sensor film for this application. One can see in samples S1-S3 that there is an uneven pressure distribution on the bone beam. The darker magenta mark to the right on
these samples indicates that there is a larger pressure or force being applied to this side of
the bone beam than to the left, where the marks are a much fainter and lighter shade of
magenta. This profile suggests that the bone beam is uneven in height across its length.
Once this region of the bone beam was sanded down, the pressure distribution became
more even in samples S4-S7. Having an even pressure distribution is important in this
application in order to validate the four point loading method.

Figure 4.11 below shows a grid of the samples which was accompanied with the
minimum, maximum, and average pressure reading for each cell in a Microsoft Excel
spreadsheet. Examples of the pressure readings provided for cells H9-G10 are shown in
Table 4.1.

Figure 4.11: Grid analysis of film samples provided by Sensor Products Inc. to measure pressure
distribution at a displacement of 40 μm.

<table>
<thead>
<tr>
<th>X Label</th>
<th>Y Label</th>
<th>Contact Area (sq.in)</th>
<th>Avg Pressure (PSI)</th>
<th>Force (lbf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>9</td>
<td>1.81E-03</td>
<td>2164.7</td>
<td>3.9</td>
</tr>
<tr>
<td>H</td>
<td>10</td>
<td>1.81E-04</td>
<td>1752.6</td>
<td>0.3</td>
</tr>
<tr>
<td>G</td>
<td>9</td>
<td>3.69E-03</td>
<td>2499.3</td>
<td>9.2</td>
</tr>
<tr>
<td>G</td>
<td>10</td>
<td>2.94E-04</td>
<td>1795.1</td>
<td>0.5</td>
</tr>
<tr>
<td>X Label</td>
<td>Y Label</td>
<td>Min Pressure (PSI)</td>
<td>Max Pressure (PSI)</td>
<td>Standard Deviation (PSI)²</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>H</td>
<td>9</td>
<td>1437</td>
<td>4021</td>
<td>670</td>
</tr>
<tr>
<td>H</td>
<td>10</td>
<td>1437</td>
<td>3202</td>
<td>412</td>
</tr>
<tr>
<td>G</td>
<td>9</td>
<td>1437</td>
<td>4816</td>
<td>928</td>
</tr>
<tr>
<td>G</td>
<td>10</td>
<td>1437</td>
<td>3365</td>
<td>382</td>
</tr>
</tbody>
</table>

Table 4.1: Pressure data provided by Sensor Products Inc. for the sample grid in Figure 4.11.

Unfortunately most of the cells include pressure readings from the edges where the film was cut, which are not readings that are desired. However, cells similar to H9 do clearly show the mark made by the plunger tip with minimal edge effects. The maximum pressure in cell H9 was found to be 4021 psi. For comparison, the maximum pressure in cell G9 was determined to be 4816 psi, though one can see from Figure 4.11 that this maximum pressure may be in the upper left corner of the cell, and not necessarily where the plunger tip made contact. Nevertheless, these two numbers differed by 18% indicating that the pressure distribution on this particular bone sample was reasonably even.

Based on the two pressure readings mentioned above, an average pressure of 4418.5 psi was used in calculations for estimating Young’s modulus for this particular bone beam using Equations 1 and 2 in Section 3.2.3. The modulus was determined to be approximately 40 GPa, which appears to be much higher than the range of reported values from other sources (∼15-20 GPa for an adult canine tibia)⁷. Since this calculation was only based on a single bone beam and is really a rough estimation of the modulus of elasticity, more bone beam samples from different sections should be tested with the sensor film if the goal of future work is to obtain more accurate measurements for the modulus.
4.3 Sample Immersion Experiments

Following successful validation of the mechanical loading system, sample immersion experiments were performed. First, several encapsulated bone beams were placed in the mechanical loading system without any loading applied. These unloaded beams would serve as the baseline control for comparison with the mechanically loaded beams in the sample immersion experiments.

Figures 4.12A-C show three of the imaged samples from the same unloaded bone beam. The left edge of each slice was on the side of the bone beam in direct contact with the sodium fluorescein solution. Thus, a high intensity of sodium fluorescein is observed in this region. Further to the right of each image, the intensity of the sodium fluorescein decreases steadily, and almost no fluorescence is detectable near the far right end of each sample.

![Image of slices taken from an unloaded bone beam after the sample immersion experiment](image)

**Figures 4.12A-C:** Images of slices taken from an unloaded bone beam after the sample immersion experiment. Camera settings on the microscope were set to a gain of 2 and exposure time of 250 ms.

The intensity profiles shown from Figures 4.12A-C were used to generate concentration
profiles using a previously-written Matlab code. An autofluorescence value was first subtracted from each average intensity value to account for the fluorescence of molecules in a bone beam prior to immersion in sodium fluorescein. Figures 4.13A-C show the normalized concentration profiles and best fit diffusion coefficient for each of these samples. The blue curve represents the average value based on the original intensity data from each image. The confidence intervals are shown by the red dash marks. The green curve is the re-calculated profile from the model equation using the best fit diffusion coefficient.
Figures 4.13A-C: Normalized concentration profiles and reported transport parameters, D for three unloaded samples from Figures 4.12A-C. The blue line shows the actual average, normalized concentration (theta) with the red dashes marking the 95% confidence intervals. The green curve is the calculated concentration profile based on the best-fit diffusion coefficient shown for each image.

The best-fit diffusion coefficient values for the three samples appear to be in close agreement with each other. However, upon further inspection of the imaged bone samples, it appears that at least two of the samples (Figures 4.12A and 4.12B) show overexposure of fluorescence at the left edge. This behavior can also be seen in the respective concentration profiles in Figures 4.13A and 4.13B. Theta, the normalized concentration, appears to increase within the first 100-200 μm of these samples. In theory, the highest concentration should be at the boundary layer, which is inconsistent with the observed data. Attempts were made to correct this behavior by eliminating more of the left edge of the sample such that the boundary layer of the sample was considered to be at the point of maximum intensity. The corrected concentration profiles and recalculated diffusion coefficient values for these two samples are shown below in Figures 4.14A and 4.14B.
Figures 4.14A-B: Corrected concentration profiles and reported transport parameters, D for unloaded samples A and B. The blue line shows the actual average, normalized concentration (theta) with the red dashes marking the 95% confidence intervals. The green curve is the calculated concentration profile based on the best-fit diffusion coefficient shown for each image.

Although the correction made to sample 2 had little effect on the best fit diffusion coefficient (<10% difference), the corrected diffusion coefficient for sample 3 increased by more than a factor of 3. This reveals a major limitation in the corrections made to these images. Making corrections to oversaturated images introduces a significant amount of bias that could affect the calculated diffusion coefficient dramatically. Instead of attempting to correct already oversaturated images, future images should be taken at smaller exposure times to reduce overexposure. Though the images will inevitably appear darker, and less detail of the osteon groups will be visible, oversaturation will be
eliminated. Concentration profiles closer to Figure 4.13C might better represent the transport of sodium fluorescein in these bone samples.

The effect of autofluorescence was also studied by changing the value of this parameter in the Matlab code for a different unloaded sample. Figures 4.15A-C show a concentration profile of the same image with different autofluorescence values subtracted from the average intensity. The autofluorescence of 35.27 in Figure 4.15B is the average intensity of a blank bone sample that was imaged at the same gain (2) and exposure time (250 ms) as the other unloaded samples. Autofluorescence values above (40) and below (30) were also used to show how much the autofluorescence value impacts the concentration profile and ultimately the value of the diffusion coefficient.
Figures 4.15A-C: Effect of autofluorescence (A-30, B-35.27, C-40) on concentration profile and transport parameter, D for different unloaded sample. The blue line shows the actual average, normalized concentration (theta) with the red dashes marking the 95% confidence intervals. The green curve is the calculated concentration profile based on the best-fit diffusion coefficient shown for each image.

As Figures 4.15A-C indicate, the smallest autofluorescence value results in the largest diffusion coefficient ($1.09 \times 10^{-8} \text{ cm}^2\text{s}^{-1}$). It appears that a larger value subtracted from the average intensity forces the profile to approach a value of 0 at a shorter distance into the sample, which would lead to a smaller diffusion coefficient. Even a relatively small change (5 intensity units) in the autofluorescence value can cause a significant difference ($\approx 20\%$) in the best fit value of the diffusion coefficient. Therefore, it is important that the autofluorescence value be obtained from the unexposed bone beam at the same gain and exposure time as the bone samples for each experiment.

Finally, the effect of setting negative intensities to a value of 0 after the autofluorescence had been subtracted out was also investigated. From a physical standpoint a negative intensity value does not make conceptual sense, since that would imply that the concentration of the solute at that particular location is negative, which is meaningless. Depending on the value of the autofluorescence however, it is possible for the intensity to be negative. In previous work, these negative values were simply set
equal to zero before being averaged\(^{10}\). Figures 4.16A-B show the same concentration profile, one with the negative intensities set equal to zero (Figure 4.16A), and the other without any other mathematical adjustments aside from the autofluorescence (Figure 4.16B).

Figures 4.16A-B: Effect of including negative intensities on the concentration profiles and transport parameter, \(D\) for unloaded sample. The blue line shows the actual average, normalized concentration (\(\theta\)) with the red dashes marking the 95\% confidence intervals. The green curve is the calculated concentration profile based on the best-fit diffusion coefficient shown for each image.

It is readily apparent that Figure 4.16B shows concentrations that are less than 0. Although negative concentrations may not make physical sense, one could argue that
simply setting any negative intensity values equal to zero would only be affecting the region near the end of the concentration profile, since these are the intensity values that are much closer if not less than the autofluorescence value. It is highly unlikely that the intensities near the beginning of the concentration profile would ever be negative. Therefore, since setting negative intensities to zero only changes a certain region of the bone sample, this mathematical processing inevitably changes the true concentration profile. However, this processing does provide a better fit to the model prediction shown by the green curve in Figure 4.16A, which always approaches a concentration of 0. Despite these two different methods, there is less than a 10% difference between the best fit diffusion coefficients shown in Figures 4.16A and 4.16B.

Figures 4.17A-C show three of the imaged samples from the same bone beam that had undergone continuous mechanical loading. Pressure was applied in the form of a sinusoidal wave with amplitude of 18 μm (maximum applied displacement of 36 μm) for a period of 24 hours while the bottom face of the bone beam was exposed to a 30 μM sodium fluorescein solution.
Comparing these images to the unloaded samples in Figures 4.12A-C, one can see that there is a much greater transport of sodium fluorescein further into the bone. Although images 4.17A-C were taken at a higher gain (8 vs 2) and shorter exposure time (25 ms vs 250 ms) than the unloaded images to further reduce any oversaturation, one can still see a great level of detail. Many of the osteon structures in these samples appear to fluoresce with the solute even beyond 1 mm along the length of the slice. In the unloaded samples, only a few osteon groups are visible with the sodium fluorescein within the first few hundred microns of the sample length. To further demonstrate that these images are indeed comparable, the average autofluorescence determined from the unexposed sample cut from the end of this beam at the current gain of 8 and exposure time of 25 ms was 32, compared to 35 for the unexposed sample cut from the different beam at the previous conditions (gain 2, exposure time 250 ms).
Concentration profiles are also shown for the loaded samples in Figures 4.18A-C to provide a quantitative comparison between the loaded and unloaded cases. Along with these profiles a best-fit transport parameter was again calculated using the diffusion model given by Equation 5 in Section 3.3.4. Although this parameter does not represent the diffusion coefficient, since both diffusion and convection are occurring in the loaded system, an increase in the value of this parameter does represent an enhancement to the transport of sodium fluorescein through the cortical bone. In order to be consistent, the parameter will be referred to as the transport parameter throughout the remaining sections.
Figures 4.18A-C: Normalized concentration profiles and reported transport parameters, D for three loaded samples from Figures 4.17A-C. The blue line shows the actual average, normalized concentration (theta) with the red dashes marking the 95% confidence intervals. The green curve is the calculated concentration profile based on the best-fit diffusion coefficient shown for each image.

One will immediately notice that these normalized concentration profiles do not approach 0 as readily as the profiles for the unloaded samples. As a result, the best-fit transport parameter for the loaded samples is much greater, on the order of $10^{-8}$ cm$^2$s$^{-1}$ compared to $10^{-9}$ cm$^2$s$^{-1}$ for the unloaded bone beams. In several of these loaded samples the concentration curve does show some sharp increases, especially within the first few hundred micrometers of the sample length. One will notice that in these regions a few of the osteons groups show a great deal of fluorescence, while the area immediately surrounding these osteons appears much darker. This observation is key to understanding how the sodium fluorescein is being transported throughout the cortical bone. A series of Volkmann’s canals connecting osteon groups allows the sodium fluorescein to move radially through the bone beam from one osteon group to another. Thus even further along the length of the sample there are still osteon groups that show fluorescence, while the majority of the area in this region appears to be unaffected.
Figure 4.19 shows the overall results for all the loaded and unloaded bone beam samples. A total of two loaded trials and two unloaded trials were completed. Unfortunately, the first loaded and unloaded trials only have 1 sample due to issues cutting the bone beam into slices. The second trials were slightly more successful. Four images were able to be obtained for the second loaded trial, and three for the second unloaded trial.

Figure 4.19 shows a plot of the average transport parameter for 5 loaded and 4 unloaded bone samples with the standard error. The average value and standard error of this parameter for the loaded samples was $3.70 \times 10^{-8} \pm 1.31 \times 10^{-8} \text{ cm s}^{-1}$, and $6.59 \times 10^{-9} \pm 2.46 \times 10^{-9} \text{ cm s}^{-1}$ for the unloaded samples. The average value of the transport parameter for the samples from loaded bone beam increased by more than a factor of 5 compared to the unloaded samples. In order to determine whether or not the unloaded
and loaded data sets are significantly different, a student’s t-test was performed. The null hypothesis was that the unloaded and loaded data sets are not significantly different. In order to reject the null hypothesis, the p value needs to be less than 0.05, otherwise there is a greater probability that the two data sets are not significantly different (i.e. the null hypothesis cannot be rejected). A p value of 0.08 was calculated, which is greater than the significance value of 0.05. Since the p value is slightly above 0.05, technically the null hypothesis cannot be rejected. There is a higher probability that the unloaded and loaded data sets are not significantly different. However, one should keep in mind that the number of samples for each data set is rather small (n=4,5), and the p value of 0.08 is very close to the value of 0.05. Therefore, one might expect that with more samples that the unloaded and loaded data sets may indeed be more likely to be significantly different. Despite the limitations of the sample size, this data does seem to provide preliminary quantitative evidence that mechanical loading does enhance solute transport in the cortical bone of the canine tibiae.

4.4 Raloxifene Study for Future Work

In a separate study, the fluorescence properties of Raloxifene were investigated for its potential usefulness as a solute tracer in future sample immersion and mechanical loading experiments. The primary goal was to see whether or not Raloxifene had well-defined excitation and emission spectra that would allow the fluorescence intensity to be accurately measured. 1 μM samples of Raloxifene in DMSO were prepared and analyzed using a Hitachi-7000 spectrophotometer in the Chemistry Department at Cleveland state University. An excitation scan was first performed as shown in Figure 4.20A. The fluorescence intensity is plotted against the excitation wavelength (nm).
Figures 4.20A-B: Excitation (A) and emission (B) spectra for 1 μM Raloxifene sample. Emission spectrum is based on an excitation wavelength of 500 nm.

Based on Figure 4.20A it appears that the maximum intensity peak begins to occur at a wavelength of approximately 500 nm. This excitation wavelength was then used to generate the emission spectrum, which is shown in Figure 4.20B. Based on the peak in Figure 4.20B, the emission wavelength was determined to occur at approximately 504 nm. Unfortunately, the excitation wavelength (500 nm) and emission wavelength (504 nm) are much too close together to prove that Raloxifene has any consistently measurable fluorescence on its own. Although it is difficult to predict the fluorescent properties of
any compound based solely on structure, the presence of the sulfur group on one of the
carbon rings in the Raloxifene structure shown in Figure 4.21 may in fact be able to
attract and hold more electrons. If these electrons were able to be emitted, perhaps a
higher emission wavelength would be observed.

Figure 4.21: Structure of Raloxifene; note the presence of a sulfur group on one of the carbon rings.

When the excitation wavelength was changed to 470 nm, the emission spectrum
revealed a peak at approximately 474 nm, demonstrating that simply adjusting the
excitation wavelength would not result in a greater difference between excitation and
emission spectra either. In comparison, sodium fluorescein, the current solute of interest,
has a much greater difference between the excitation (494 nm) and emission (521)
wavelengths.

Additional attempts were made to see if the solution properties had any effect on
the fluorescence of Raloxifene. An aliquot of 1M NaOH was added to shift the pH of the
solution to about 10 and bring the NaOH concentration to 10 mM in the Raloxifene
sample. Figures 4.22A-B show the excitation and emission spectra for this sample.
Figures 4.22A-B: Excitation (A) and emission (B) spectra for Raloxifene aliquot with 1 mM NaOH addition. Emission spectrum was generated based on an excitation wavelength of 470 nm.

Although the addition of NaOH did appear to increase the magnitude of the fluorescence intensity by over an order of magnitude (see the y-axis in Figures 4.20A-B compared to 4.22A-B), the excitation and emission spectra were too close together, once again at 470 and 474 respectively. Therefore, it was determined that Raloxifene would not serve as a suitable fluorescent tracer in future applications.
CHAPTER V
DISCUSSION

The techniques for measuring the center deflection of the bone beam as well as the pressure on the bone during loading came with several limitations. The current mechanical loading system does not easily accommodate bone beams of different heights. Shims were used to adjust the plate height if needed, but these shims are at least 50 μm in thickness. Therefore, if a bone beam does not fit exactly into the system, there will inevitably be a certain amount of pre-loading that will occur before any actual displacement is applied by the actuators. This additional deflection and pressure cannot easily be taken into account, and may provide more deflection than desired.

Additionally, the piezo actuators are not able to detect the force or pressure on the bone beam, so external methods had to be used in order to estimate these values. Although force transducers were unable to be placed on the bone beam due to the tight spacing constraints within the system, a pressure indicating sensor film was utilized to estimate the pressure exerted on the bone beam by the plunger tips. However, problems with how the film samples were scanned led to considerable errors in the pressure measurements.
It does appear that this limitation can be fixed and that the sensor film can be used to obtain more accurate and precise results in future experiments.

There were several significant difficulties with the experimental setup that resulted in a small number of samples for both loaded and unloaded experiments. In previous work, the sample holder that was used to cut bone slices was made for a bone that had been encapsulated in a dental resin block of predetermined size\textsuperscript{10}. Unfortunately, these encapsulated beams would not fit in the mechanical loading system, and an alternative method of sealing the bone had to be devised. When it came time to cut slices, the sealed bone beams did not fit well into the sample holder on the slow-speed saw, and had to be awkwardly placed between the two main pieces. Additionally, another bone beam had to be placed on the other side of the sample holder to prevent the bone beam of interest from sliding out of position. This made cutting thin, uniform slices extremely difficult. Most of the samples had to be further sanded down in order to make the slices more even. The additional sanding seemed to smear many of the samples even after successive washings with PBS, and the detail and quality of some of these images suffered greatly. Samples that were too thick, too uneven, or not clear enough were not evaluated any further after images were taken, which demonstrates the lack of consistency when it comes to being able to obtain usable samples.

One major experimental design limitation with the mechanical loading system also led to a smaller number of possible samples that could be obtained from the loaded bone beams. It was necessary to remove thin sections of the silicone sealant so that the plunger tips could be in direct contact with the bone and achieve the maximum displacements possible. However, these slits allowed open spaces for the sodium
fluorescein solution to enter from the top surface of the bone. Since it is desired to measure the transport of the tracer from the bottom face of the bone only, having sodium fluorescein enter from both the top and bottom faces conflicted with the major assumption of the transport model, and thus made slices near these regions unusable for analysis. Attempts were made to keep the solution level just below the top surface of the bone beam, but there were instances where the solution would wick onto the stabilizer piece directly on top of the bone beam and bring the sodium fluorescein where the plunger was in contact with the bone beams. Usually samples that were taken within 100-200 μm of the plunger tips were discarded because of this issue. Visual inspection of the samples closest to the center of the bone beam demonstrated that they were unaffected and thus were able to be used for quantitative analysis.

Another limitation arose when imaging different bone samples on different days. The gains and exposure times were varied for the different experiments, as the methodology was being fine-tuned. A better experimental design would have been to take images of unloaded and loaded samples at the same gain and exposure time. The best comparison could be made if both loaded and unloaded samples were imaged at the same time, though this would require double the time for cutting and imaging the samples.

Although the preliminary results reported here do show that the effective diffusivity is higher in the loaded samples (was 3.70x10^{-8}±1.31x10^{-8} cm^2 s^{-1}) compared to the unloaded ones (6.59x10^{-9}±2.46x10^{-9} cm^2 s^{-1}), it is interesting to note that prior work reports the effective diffusivity of a 30 μM solution of sodium fluorescein in canine tibia to be approximately 3x10^{-8} cm^2 s^{-1}, which is much closer to the loaded cases and
significantly greater than the unloaded cases\textsuperscript{10}. Although these results seem inconsistent, there are a few differences between the sample immersion experiments performed several years ago and those used in the present work. The most likely reason for the apparent difference in effective diffusivity values has to do with the age of the sample. The previous work was performed with bone beams that were harvested from the tibia and used in the sample immersion experiments within the same year. The samples in the present work have been stored since 2007, almost an entire 8 years before they were utilized in the current experiments. During that time it is likely that a breakdown of the bone matrix may have gradually occurred, releasing proteoglycans, proteins, hydroxyapatite, and other large molecules into the interstitial solution within the Volkmann and Haversian canals of the bone. The presence of these molecules in the solution would obstruct the sodium fluorescein molecules from moving as easily through the bone beam, leading to a slower transport rate and a smaller effective diffusivity.

It should also be noted that these bone beams underwent continuous mechanical loading for a period of 24 hours, which may not be representative of the forces on a bone beam in a canine or any mammal under normal conditions. The rationale behind the continuous loading for this period of time was to see whether or not mechanical loading could cause a measurable change to the effective diffusivity compared to the unloaded bones. Thus, it made sense to first test the bone beams at the maximum loading parameters. If solute transport was not observed to be enhanced at all by these mechanical loading conditions, then it is highly likely that no measurable difference in the effective diffusivity would be measured, even with loading parameters closer to more physiologically normal conditions.
Despite the limitations described above, the preliminary results from the current research do indicate that there is a measurable difference in the effective diffusivity of sodium fluorescein between loaded and unloaded canine tibia cortical bone. Although this enhancement of solute transport due to mechanical loading has been theorized in literature, most of the quantitative evidence published by groups such as Wang et al. has focused on solute transport within individual lacunae or Haversian systems\textsuperscript{19,25}, while this research has investigated the transport of sodium fluorescein in the radial direction across the entire cortical bone tissue of the canine tibia. It comes as no surprise that values for the effective diffusivity that were determined in these experiments are two to three orders of magnitude ($10^{-8}$-$10^{-9}$ cm$^2$s$^{-1}$) smaller than those determined by Wang et al. ($10^{-6}$ cm$^2$s$^{-1}$), considering that transport of a solute through the entire dense, mineralized cortical bone matrix is expected to be slower than through individual canals. More quantitative data is still needed to definitively show the affect that mechanical loading provides to the effective diffusivity of sodium fluorescein in cortical bone, but the initial findings that the transport is enhanced by applied loading is consistent with Wang et al. and other groups performing similar research.
CHAPTER VI
CONCLUSION AND RECOMMENDATIONS

6.1. Conclusions

There were several important goals in the proof-of-concept work for the mechanical loading system and sample immersion experiments. First of all, a validation procedure had to be performed in order to ensure that the mechanical loading system could provide enough force to deflect the bone beams a measurable distance. The deflection was visually observed with the aid of a high speed camera, and distance calibrations allowed for accurate measurement of the center deflection using ImageJ. Additionally, a pressure indicating sensor film was used to provide estimates of the pressure on the bone beam during loading.

Once the mechanical loading system was proved to be stable and functioning properly, sample immersion experiments from previous work were modified based on the current experimental setup. This was accomplished by encapsulating the bone beams in a smaller amount of silicone sealant so that the bone beam could fit properly into the mechanical loading system. Sealed bone beams were immersed in 3 mL of a 30 μM sodium fluorescein solution for a period of 24 hours. Unloaded bone beams were first
used as a baseline for comparison to bone beams that were subject to continuous applied
loading. The effect of mechanical loading on the transport of sodium fluorescein through
the canine tibiae bone beams was observed qualitatively by comparing the images of the
bone beam slices as well as quantitatively by comparing the concentration profiles and
best fit effective diffusivity values which were determined for both unloaded and loaded
bone beams. Finally, fluorescence spectroscopy studies for Raloxifene showed that this
molecule did not have fluorescent properties that could be utilized for future application
in these and similar experiments.

The following conclusions present the specific findings from the methods described
above:

1. The center deflection of bone beams was successfully observed and
measured in the mechanical loading system during applied loading. Two
different analytical methods of the images taken found a strong linear
correlation between the center deflection of the bone beam and the applied
displacement of the piezo actuator.

   a. The first method used the merged images of the bone beam taken at
both unloaded and loaded states, and measured the overlap region of
the two images. The slope of the best fit line in a plot of bone beam
center deflection vs. applied displacement for this method was
determined to be $1.06 \pm 0.02$. The average percent error between the
applied deflection as the true value and the measured deflection was
6%. 

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b. The second method measured the distance that the maximum change in intensity moved after a displacement was applied, which was shown to occur at the boundary layer between the bone and background. The slope of the best fit line in a plot of bone beam center deflection vs. applied displacement for this method was determined to be 0.93 ± 0.01. The average percent error between the applied deflection as the true value and the measured deflection was 7%.

2. The pressure on the bone beam at the point of contact from the plunger tips was estimated using a pressure indicating sensor film. Initial readings indicated that the pressure on the bone beam at these locations was approximately 4400 psi. From these pressure readings, a Young’s modulus of approximately 40 GPa was calculated for this particular bone beam, which compares to previously reported values of 15-20 GPa for canine tibia. 

3. Preliminary sample immersion experiments show an enhancement of sodium fluorescein transport in canine tibiae bone beams due to mechanical loading both qualitatively and quantitatively. Bone samples from loaded bone beams showed more osteon groups fluorescing further along the radial distance of the sample compared to the unloaded counterparts. Moreover, the average effective diffusivity for the loaded samples was determined to be 3.70x10^{-8} ±1.31x10^{-8} cm²s⁻¹, more than five times greater than the average effective diffusivity of the unloaded samples, which was found to be 6.59x10^{-9} ±2.46x10^{-9} cm²s⁻¹.
4. The excitation and emission peaks for Raloxifene were found to be too close together to prove that Raloxifene had a measurable fluorescence. Therefore, this compound cannot be utilized in future loading experiments.

6.2. Recommendations

The following recommendations are made for the continuation of this research project:

1. One of the major limitations of the mechanical loading system was the fact that shims had to be inserted in order to provide enough clearance for the bone beam to fit without too much of an applied preload. This may be accomplished in one or both of the following ways:
   a. Further modifications may be made to the mechanical loading system that allows for samples of different heights to fit easily within the system. Currently the use of shims will only allow bone beams of certain height to fit in the system.
   b. Changes to the procedure for cutting bone beams from the tibiae sections can be made to allow for better control over the dimensions of the bone beams.

2. The pressure indicating sensor film should continue to be utilized in order to provide a large data set for measuring the pressure on the bone beams during mechanical loading. If this data proves to be important, a calibration of the pressure as a function of the applied displacement could be developed as well.
3. The number of samples from the loaded bone beams was limited due to the slits that were cut out for the plunger tips to contact the bone beam, which allowed for sodium fluorescein to enter through the top face of the bone. Different materials similar to cement should be investigated to see whether or not these surface can be closed while still allowing the plunger to provide enough deflection to the bone beam.

4. Additionally, the small number of samples from both loaded and unloaded bone beams was the result of difficulty cutting bone slices since the bone beams did not fit well into the sample holder. It is recommended that a new sample holder for the slow-speed saw be designed that will allow more flexibility in the size of bone beams that are to be further cut into slices for imaging.

5. As shown in the Results section, the subtracting of autofluorescence and the adjustment of negative intensities does affect the best fit effective diffusivity. More guidance and a standard protocol should be developed for making adjustments to images so that more confidence can be achieved in determining the value of the effective diffusivity and more consistency can be found in comparing results from one experiment to another.

6. The present model for measuring the effective diffusivity does not include any convection term or similar parameter for quantifying the contributions of transport from loading. It is recommended that a model that includes a convective term be investigated for its potential use in these experiments.
WORKS CITED


   http://humanresearchroadmap.nasa.gov/Evidence/reports/Bone%20Fracture.pdf


24. Vitamin D (Calcitriol) structures: Image obtained on 7 August 2015 from http://examine.com/supplements/vitamin-d/
Appendix A: Mechanical Loading System Assembly Protocol

I. Scope
   1. The purpose of this document is to provide instructions for assembling the mechanical loading system.

II. Application
   1. The mechanical loading system is used for four point bend testing on bone beam samples as well as sample immersion experiments both with and without loading.

III. Safety
   1. It is important that all components (especially piezo actuators) are stable and secure within the system. Damage to the actuators could result in mechanical failure or serious injury due to shock if the electrical components are loose or frayed. See the safety section in the E-516 User Manual for more details.

IV. Procedure
   A. Base Assembly (Bottom Plate – Plunger Pieces)
      1. Place the four 3” screws through the holes in each corner of the base plate as shown in Figure 1. Place a 0.5” spacer on each screw as shown below.

      ![Figure 1: Base Plate with Screws and Spacers](image)

      2. Position the acrylic 6 well plate so that it fits securely into the “well” of the base plate, as shown in Figure 2.

      ![Figure 2: Acrylic 6 Well Plate](image)
3. Place the three sample holders in the well plate as shown in Figure 3.
   1. One sample holder has a thin section removed from the bottom to allow for better viewing of the bone beam. Additionally, one of the ports on the acrylic 6 well plate was enlarged to 5/16” for the same purpose. Place that sample holder in this well as shown in Figure 4.

4. Place the bone beam on the modified sample holder in the well with the 5/16 ” port as shown in Figures 5 and 6.

5. Place the stabilizer piece on top of the bone and each sample holder (including the two without the bone beams as shown in Figure 7).
6. Place the thin plate with the three open holes through the large screws and on top of the acrylic well plate as shown in Figures 6 and 7. The top of the acrylic well plate should fit securely in the bottom face of this plate.

![Figures 6 and 7: Securing the Acrylic Well Plate](image)

7. Place a 40 durometer rating silicone O-ring (1.239” diameter x 0.07” thickness) in each of the wells on the top plate as shown in Figure 8.

![Figure 8: O-rings](image)

8. Place the plunger piece in Figure 9 on top of the O-ring as shown in Figure 10. Make sure that the plunger is stable and level on the bone beam so that forces are applied evenly during four point bend testing.

![Figures 9 and 10: Positioning the Plunger Piece](image)
9. Add additional spacers as needed on top of the plate as shown in Figure 12. These spacers are necessary to keep the actuators, plunger, and bone beam all in contact since the bone beams are often of variable height.

![Figures 11 and 12: Adding Spacers for Proper Component Fitting](image)

B. **Top Section Assembly (Piezo Actuators)**

1. It is easier to assemble the top section with the piezo actuators separately from the base components.

2. Place the three 3” screws through the bottom of the 0.5” thick “base” plate along with the three 2” spacers as shown in Figure 13.

![Figure 13: Assembly of base plate for top section](image)

3. Place the movable component of each actuator in the appropriate hole of the base plate as shown in Figure 14.

![Figure 14: Piezo Actuator Placement](image)
4. Each actuator also contains a small screw insert as shown in Figure 14. This piece is used to pivot the top part of the actuator against the bottom of the top plate (see Figures 17 and 18).

5. Place the top plate above the actuators as shown in Figures 15 and 16.

6. Use the screw inserts to securely fit each actuator as shown in Figure 17. Tighten the top plate using three wing nuts as shown in Figure 18.

7. Place the top section above the bottom section that was assembled earlier and tighten the four wing nuts as shown in Figure 19.
Appendix B: E-516 Controller Unit Protocol

V. Scope
   1. The purpose of this document is to provide instructions for the basic operation of the E-516 Controller Unit and software using LabVIEW.

VI. Description
   1. The E-516 Controller Unit is used to control the displacement (0-40 μm) of three piezo actuators by changing the voltage (-1000-0 V).

VII. Safety
   1. It is important to handle all components of the E-516 Controller Unit properly in order to avoid shock or serious injury. Make sure that the wires on the piezo actuators are untangled and secured.

VIII. Procedure
   A. Basic Assembly of the E-516 Controller Unit
      1. The E-516 Controller Unit and piezo actuators (in the mechanical loading system) are shown in Figure 1. Each actuator has its own designated channel that has been marked on the actuator as shown in Figure 2.

         Figures 1 and 2: E-516 Controller Unit and piezo actuators in mechanical loading system

      2. Connect the top wire (furthest from the moving component) on each actuator to the appropriate PZT High Voltage port as shown in Figure 3. Connect the bottom wire (closest to the moving component) on each actuator to the appropriate Servo sensor port as shown in Figure 4.

         Figures 3 and 4: Connecting the wires to the PZT High Voltage (3) and Servo Sensor (4) ports on the E-516 controller unit.
3. Once all of the actuators have been connected, turn on the E-516 Controller Unit by locating the power switch on the back of the controller as shown in Figure 5. The Display/Interface panel should now be lit and showing the voltage (V) and displacement (μm) for each actuator as shown in Figure 6.
   1. Since the controller is not being run with any software yet, the upper left hand corner should read OffLine. In this mode, commands can only be made manually by using buttons directly below the display screen (yellow box) in Figure 6.
   2. It is **not recommended** to use this manual method for sending commands since software has been provided.

4. Connect the female end of the RS232 cable to the RS232 port on the lower right corner of the E-516 controller unit as shown in Figures 7 and 8.

5. Connect the other female end of the RS232 cable to the TrendNET USB adapter as shown in Figure 9.
   1. Note: This adapter is included so that the RS232 cable can be connected to the USB port. Although the current computer does come with an RS232 port, most computers will not have this port available should the system need to be updated for any reason.
2. The RS232 cable can be connected to the RS232 port on the computer itself as shown in Figure 10, but the configuration setup with the software will be slightly different as detailed later.

![Figures 9 and 10: Connecting the RS232 cable to the computer via USB adapter (9) or RS232 port (10)](image)

6. Once all of the connections are made, turn on the computer to begin running the software. Details in next section.

B. Configuration Setup
   1. Turn on the computer and click on the *My Computer* icon on the desktop.

![Figure 11: Opening *My Computer* from the desktop](image)

2. Click on the *Local Disk (C:)* icon in the *My Computer* directory.

![Figure 12: Opening *Local Disk (C:)* from the *My Computer* directory](image)
3. Click on the *Program Files* Folder in the *Local Disk (C:)* directory.

![Figure 13: Opening the Program Files folder from the Local Disk (C:) directory](image)

4. Click on the “Show Files” link to display additional folders in the Program Files directory.

![Figure 14: Revealing Additional Folders under Program Files](image)

5. Click on the *PI* folder in the *Program Files* directory.

![Figure 15: Opening the PI folder from the Program Files directory](image)
6. Click on the *E-516* folder in the PI directory.

![Figure 16: Opening the E-516 folder from the PI directory](image)

7. Click on the *E516_GCS_LabView* folder in the *E-516* directory.

![Figure 17: Opening the E516-GCS-LabView folder from the E-516 directory](image)

8. Right Click on the *E516_Configuration_Setup* icon to run the program in LabVIEW.

![Figures 18 and 19: Opening the E-516 Configuration Setup in LabVIEW](image)
9. The Configuration Setup virtual instrument (vi) should open as shown below. Change the RS232 settings to the following: Portnumber – COM3, Baudrate – 115200 as shown in Figures 20 and 21.

   1. If the RS232 cable is plugged in directly to the RS232 port on the computer (not through the TRENDnet USB converter), change the Portnumber to COM1.

10. Click on the Run icon (arrow pointing to the right in Figure 22) located in the upper left hand corner to initiate the configuration setup.

11. The Identification box will now display the communication information (red box in Figure 23), and each actuator will be set to a displacement of 20 μm (yellow box in Figure 23; Display panel in Figure 24).
12. Close the E516_Configuration_Setup.vi window since it is now confirmed that the LabVIEW communication with the E-516 controller is working.

13. Right click on the **PI Terminal** icon in the *E516_GCS_LabView* folder and open it with LabVIEW.

![Figure 25: Running the PI Terminal program in LabVIEW](image)

14. The PI Terminal vi will open as shown below in Figure 26. Click on the *Run* arrow in order to change the Portnumber and Baudrate settings in the Select Interface Parameters window shown in Figure 27.

![Figures 26 and 27: Configuring the PI Terminal settings](image)
15. A new screen will open that will allow the user to select the RS232 Portnumber and Baudrate. Change the Portnumber to \textit{COM3} using the dropdown menu. Manually input 115200 for the Baudrate. Click on the \textit{OK} button in the upper right hand corner once the changes have been made.

1. Once again, if the RS232 cable is connected directly to the computer’s RS232 port, select \textit{COM1} for the Portnumber.

Figures 28 and 29: Changing the RS232 portnumber and baudrate

16. Commands can now be sent to the piezo actuators using the PI Terminal Send Window.

1. Basic commands will be shown in the next section of this protocol. For a complete list of command instructions and notations, refer to the E-516 Controller User Manual.

C. Basic Commands using the E-516 Controller Unit: Changing Displacement

1. Type in the command \textit{ONLI} in the first line of the Send window and press the Enter key. This will ensure that the RS232 system is online and commands can be sent to the actuators.

Figure 30: Online command
2. In order to change the displacement of an actuator, start with the command MOV, followed by the actuator name (Channel 1 – A, Channel 2 – B, Channel 3 – C) and displacement (a number between 0 and 40 representing displacement in μm) as shown in Figure 31.
   1. This step may be done for any or all of the actuators at the same time. Figures 31 and 32 show an example of moving all three actuators to a displacement of 2 μm at the same time.

3. Displacements in the range of 0-35 μm are recommended since the maximum capability of each actuator is 40 μm.

D. Basic Commands using the E-516 Controller Unit: Implementing a Sine Wave
   1. The code below in Figure 33 executes a sine wave, 0-36 μm, period of 0.5 sec (f = 2 Hz), 50 cycles, then stops at position 0 μm for the actuator in Channel 3.

2. See the E-516 User Manual for complete instructions on how to create a wave, or modify the parameters (period, amplitude, etc) of a current wave.
Appendix C: Protocol for the ImperX Camera System

IX. Scope
   1. The purpose of this document is to provide instructions for the operation of the ImperX camera system using StreamPix.

X. Application
   1. The ImperX camera system is used to monitor the displacement of a bone beam under mechanical loading.

XI. Safety
   1. It is important to make sure that the camera is stable on the boom stand to obtain the highest quality images. Be careful when loosening the clamps on the boom stand to adjust the camera position.

XII. Procedure
   A. Basic Setup of the Boom Stand
      1. The ImperX camera can be attached to a boom stand to provide for stable horizontal and vertical movement as shown below in Figure 1.

      ![Figure 1: ImperX camera setup on boom stand](image)

      2. In order to adjust the vertical height of the camera, loosen the clamp below the vertical base of the boom, and turn the black knob on the boom to the left while holding the boom securely (Figure 2). Carefully adjust the height of the boom, and tighten both the knob and the clamp when finished. Mark the position as needed for future reference.

      ![Figure 2: Vertical positioning adjustment](image)
3. Use the knob shown in Figure 3 on the right side of the boom stand to adjust the horizontal position of the camera.

![Figure 3: Horizontal positioning adjustment](image)

4. Check to make sure that the camera is level prior to taking images as shown in Figure 4.

![Figure 4: Ensuring the camera is level](image)

5. Place two metal blocks (one on either side of the camera as shown in Figure 5) in order to keep the camera stable and prevent it from moving with vibrations.

![Figure 5: Stabilizing the ImperX camera system](image)
6. Ensure that the power cord and frame grabber are attached properly as shown below in Figures 6 and 7.

Figures 6 and 7: Power and frame grabber connections

7. Adjust the magnification on the camera as shown in Figure 8. For the purposes of this validation, use the highest magnification (4X). If the image is not in focus, adjust the horizontal position of the camera or move the sample until the image is in focus.

Figure 8: Adjusting the camera magnification

8. Add additional lighting as needed in order for the camera to be able to acquire an image of the sample as shown in Figures 9 and 10.

Figures 9 and 10: Additional lighting
B. Capturing Images Using StreamPix

1. When the computer is turned on, the Device Manager application will open as shown below in Figure 11. Click Automatic to initiate the Frame Grabber configuration.

![Figure 11: Configuring the frame grabber](image1)

2. Once the update finishes, click Yes to close the application.

![Figure 12: Completing communication setup](image2)

3. Click on the LYNX Configurator icon on the desktop. An application will open for identification of the camera as shown in Figure 13. Make sure that Camera IPX-VGA210LMCN on Port 00 is highlighted and click on OK. When the program opens, the default camera settings will be displayed as shown below in Figure 14.

![Figures 13 and 14: Opening the LYNX Configurator program](image3)
4. The current settings (Speed – 29.92 fps, Exposure 33420 μSec, Taps – Single, Depth – 10 Bits, LUT – User 1) shown in Figure 15 should be used for most applications.

5. To choose from different frame grabber settings that have been previously saved, click on the File dropdown menu and select the Load from File option as shown in Figure 15. Select the file highlighted in blue in Figure 16 (30 frames per second speed) and click Open. Exit the LYNX Configurator once these settings are updated.

6. Click on the StreamPix icon on the desktop to launch the image viewing software.

7. If the user is unable to view any image and the main window is in “screensaver” mode as shown in Figure 17, it is likely that the appropriate frame grabber is not selected. Under the Hardware dropdown menu, move the cursor over the Coreco menu and select the X64 CL / X64 CL IPro frame grabber as shown in Figure 17.

Figures 15 and 16: Changing the camera settings by loading previously saved files

Figures 17 and 18: Loading the frame grabber in StreamPix
8. To create a new file or sequence of saved images or recordings, select *New Sequence on Disk*... from the *File* dropdown menu as shown below in Figure 19. Choose a file name and save the sequence on Data Drive (E:).

![Figure 19: Creating a new sequence in StreamPix](image)

9. The file name and location should now appear in the upper left hand corner of the StreamPix window as shown in Figure 20.

![Figure 20: Saved sequence](image)

10. If the viewing window is still black, apply additional lighting (see Figures 9 and 10) so that the camera picks up the sample as shown in Figure 21.

![Figure 21: Sample in view with lighting applied](image)
11. To snap a single image, click on the camera button shown in Figure 22.

![Figure 22: Snap a single image](image)

12. To record a video, click on the red *Record* button shown in Figure 23.

![Figure 23: Record a video](image)

13. To play back a sequence, click on the tape button shown in Figure 24.

![Figure 24: Playback mode](image)

14. To export a sequence as a set of images, Select *Full Sequence* from the dropdown menu as shown in Figure 25.

![Figure 25: Exporting sequence](image)

15. Once the images have been exported to the appropriate file, save the sequence and close the StreamPix program.
Appendix D: Incubator Setup for Mechanical Loading System Protocol

XIII. Scope
1. The purpose of this document is to provide instructions for setting up the mechanical loading system in the incubator.

XIV. Application
1. The mechanical loading system is used for four point bend testing on bone beam samples as well as sample immersion experiments both with and without loading. Experiments are performed in the incubator at 37 °C to simulate normal physiological conditions.

XV. Safety
1. It is important that all components (especially piezo actuators) are stable and secure within the system. Damage to the actuators could result in mechanical failure or serious injury due to shock if the electrical components are loose or frayed. See the safety section in the E-516 User Manual for more details.

XVI. Procedure
A. Basic Assembly
1. Once the mechanical loading system has been assembled, place the entire system on the top shelf of the incubator as shown in Figure 1.
   1. Make sure that the actuators wires are loose and clearly separated from one another.

   ![Figure 1: Mechanical loading system in the incubator](image)

2. Locate the port near the back of the incubator as shown in Figures 2 and 3.

   ![Figures 2 and 3: Interior and exterior view of the incubator port](image)
3. Remove the black ring and rubber stopper as shown in Figure 4.

![Figure 4: Opening the Incubator Port]

4. Thread the wires of each actuator through the port as shown in Figures 5 and 6.
   1. Repeat this step separately for each actuator so that it is easier to keep track of the wires when connecting the actuators back to the E-516 Controller Unit (see next step).

![Figures 5 and 6: Feeding the actuator wires through the incubator port]

5. Connect the wires of each actuator to the designated port on the E-516 Controller Unit as shown in Figure 7.

![Figure 7: Connecting Actuators to E-516 Controller Unit]
6. Once all of the actuators have been connected, gently place the rubber stopper back into the incubator port as shown in Figure 8. This will allow for better temperature control inside the incubator during testing.

Figure 8: Closing the incubator port

7. Close the incubator door when finished. The user may notice that the temperature on the front display may be a few degrees lower than 37 °C as shown in Figure 9. Wait a few minutes until the temperature begins to rise again before starting any experiments.

Figure 9: Temperature readings on the incubator system
Appendix E: Application and Use of Pressure Indicating Sensor Film

XVII. Scope
1. The purpose of this document is to provide instructions for using the pressure indicating sensor film provided by Sensor Products Inc.

XVIII. Application
1. The pressure indicating sensor film can be used to measure the pressure on bone beams in the mechanical loading system.

XIX. Safety
1. Be careful handling the rotary cutter when cutting the film into smaller pieces. Make sure that the blade cover is engaged whenever the cutter is not in use.

XX. Procedure
A. Preparing Pressure Indicating Sensor Film Samples
1. Align the pressure indicating sensor film on the cutting mat as shown below in Figure 1a. Place a ruler over the film where the cut is to be made as shown in Figure 1b.

Figures 1a and 1b: Pressure Indicating Sensor Film

2. Disengage the blade cover on the rotary cutter as shown in Figure 2.

Figure 2: Rotary Cutter
3. Place the blade of the rotary cutter against the ruler on the cutting mat as shown in Figure 3.

![Figure 3: Aligning the Rotary Cutter](image)

4. Cut the pressure indicating sensor film as shown in Figure 4. Be particularly careful to make cuts evenly so that only a small amount of pressure is applied at any given point. Figure 5 shows examples of even (top) and uneven (bottom) cuts on the film.

![Figures 4 and 5: Thin strip of film cut; Example of an uneven (top) and even (bottom) cut](image)

5. Engage the blade cover on the rotary cutter when finished cutting as shown in Figure 6.

![Figure 6: Engaging the blade cover on the rotary cutter](image)
B. Application: Pressure Measurements on Mechanically-Loaded Bone Beams

1. Place a piece of the pressure indicating sensor film on the bone beam in the sample holder as shown in Figure 7. The user may choose to gently tape the pressure film to the bone to keep the film aligned.

![Figure 7: Applying the pressure sensor film to the bone beam](image)

2. Assemble the remainder of the mechanical loading system as described in the protocol provided.

3. Apply a displacement of 30 μm to the actuator above the bone beam. The actuator will push the plunger against the pressure indicating sensor film and bone beam.

4. Remove the activated film from the mechanical loading system and observe the color change near the points were the plunger tips were in contact with the film as show on the samples in Figure 8.

![Figure 8: Pressure gradient on film samples in mechanical loading system](image)
5. Pressure readings may be obtained from the degree of activation as indicated by the color brightness. Samples may be sent to Sensor Products Inc. for results, or may be analyzed by the user if a scanner is available.
Appendix F: Protocol for Use of HITACHI F-7000 Fluorescence Spectrophotometer
I. Excitation Scan, Emission Scan, and Quantitation using Cuvettes
(Rev. 2012-0625)

CAUTIONS FOR LAB SAFETY
a. Do not bring food / beverage, food / beverage containers, or trash cans into the labs
b. Do not leave your items in the lab when you are not currently working here
   Move your items into your teaching / research labs or the locations assigned to you
c. Have to wear gloves when handling toxic, caustic, and unknown materials
d. Have to wear safety goggles when handling chemicals
e. Label the containers or holders of your samples / reagents (even water) with: the formal
   name of the contents (e.g. methanol instead of MeOH), your full name, your faculty
   supervisor’s name, and the date
f. Handle your wastes properly and immediately according to the rules
g. Lock the door when leaving the lab even if there are people in the next door
h. Close the hood fully when not handling items inside it

CAUTIONS FOR USE OF INSTRUMENTS WITHIN INSTRUMENTATION LABS
a. Must receive training and permission from Dr. Xiang Zhou for use of the instruments
b. Book the Instrument in advance (particularly for classes) to avoid conflicts
c. Cancel reservation in advance if you will not use it
d. Have to do cleaning and organization before start, during use, and in the end
e. Have to follow the protocol

CAUTIONS FOR INSTRUMENTATION COMPUTERS
a. Do not install any programs without the Manager’s approval
b. Do not put your files / folders on the PC Desktop; put them in the directories specified in
   the protocol
c. Do not use USB driver to backup data; use CD or DVD
d. Back up your useful data every time in the end

CATIONS FOR F7000 Fluorescence Spectrometer
a. Follow the procedure in the protocol to: turn on PC, open the program, and then turn on
   the instrument at start; close the program, turn off the lamp, and then switch off the
   instrument in the end
SECTION I. BEGINNING

1a. **Cleanup and organize** the working area
1b. **Sign in the logbook** first every day before start

2. **Turn on the PC; click the Hitachi icon to log in the computer**
3. **Switch on the instrument after** doing Step 2 (the both indicative lights are on normally)
4. **Generate a data folder for you under** C:\Hitachi Service\ (do not put folders or files on the Desktop)
   Name your folder as: YourFullName-FacultySupervisorName
5. **Open the FL Solutions Program; wait for initializing** (yellow) and get to **Ready** (green) status
6. **Let the Xenon lamp warm up for about 30 minutes**
7. **Place the cuvette containing your sample (0.7 ml minimum)** in the sample holder

**F1 for help**
SECTION II. EXCITATION WAVELENGTH SCAN

8. Generate a Method for Excitation Scan: Click Method button (Right-top) to open the Method Window, then

   a. General Tab
      Measurement: Wavelength Scan
      Operator: Your Name
      Accessory: leave blank
      Use sample table: unchecked
      Save (Save as) the method in your folder C:\Hitachi Service\YourFolder

   b. Instrument Tab
      Scan mode: Excitation
      EX Slit: 5.0 nm
      Data mode: Fluorescence
      EM Slit: 5.0 nm
      EM WL: 0 nm (to determine excitation wavelength)
      PMT Voltage: 400
      EX Start WL: i.e. 200 nm
      EX End WL: i.e. 900 nm

      □ PMT Voltage-0-1000 V
      □ Corrects Spectra
      □ Shutter Control
      Response: 0.5s; Replicates: 1-3
      Scan Speed: 2400 nm/min; Delay: 0s

   c. Monitor Tab
      Y Axis: Max: 1000; Min: 0
      Open data processing window after data acquisition: Checked

   d. Processing Tab: Do nothing

   e. Report Tab
      Output: print report or transfer data to Excel
      Check all print items
      Select desired font and size
      Data Start / Data end: the same as EX Start WL and EX End WL

   f. Save the parameters
      Go back to General Tab: Save; click Ok (instrument adjusts the parameters automatically)

9. DEFINE SAMPLE
   Click Sample button (Right – the 2nd) to Open Sample Window, then

      Enter Sample name
      Click Select button then specify the your folder to save your data
      Enter file name, then save
      Check Auto file for automatic saving
      Click OK
10. Open the Shutter: Click Open button (Right-bottom) if the shutter is closed (shown in Spectrometer Window)

11. Pre-Scan: Click Pre-Scan button (Right-the 3rd) to do the pre-scan

12. Measure Sample: Click Measure button (Right-the 4th) to start measurement
   Click Stop sign to halt the measurement

SECTION III. EMISSION WAVELEGNTH SCAN

13. Generate a Method for Emission Scan: Click Method button (Right-top) to open the Method Window, then
   a. General, Monitor, Processing, Report Tabs: do the same as Step 8
   b. Instrument Tab
      Scan mode: Emission
      EX WL: Enter the excitation wavelength determined in Section 2
      EM Start: 200 nm
      EM End: 900 nm
      Other parameters: the same as 8b
   c. Save the parameters: Go back to General Tab: Save; click Ok

14. Perform “Define Sample”, “Open the Shutter”, “Pre-Scan”, and “Measure Sample” as Steps 9-12

SECTION IV. QNANTITATION

15. Generate a Method for quantitation: Click Method button (Right-top) to open the Method Window, then
   a. General tab
      Measure: Photometry
      Save the method in your folder
   b. Quantitation tab
      Quantitation type: Wavelength
      Calibration type: 1" order
      Number of wavelength: 1
   c. Instrument tab
      Fluorescence
      Wavelength mode: Ex WL Fixed
      Ex / EM values: enter the values determined in Section II and III
      d. Standards: type in the concentrations and names of calibration standards (e.g. S1, S2)
   e. Save the method: go back to General tab to save the method in your folder

16. Perform measurement
   a. Define Sample: refer to Step 9
   d. Measure Standard: click Measure button, and then follow the instruction
   c. Measure Sample: click Sample (F4) button after putting the sample in the Sample Cell
   d. End the Series: click End (F9) to end the series

SECTION V. FINISHING
17. Exit the FL Solutions Program
   a. Click on File menu, select exit command
   b. When the popup Window appears, click “Close the lamp, and then close the monitor window”
   c. Back up your data as needed

18. Switch off the fluorescence spectrometer and shut down the PC

19. Must clean up and organize the working area:
    Do not leave your own items in the public area
    Put the Facility and your items in assigned locations
    The desk, floor, cabinet, refrigeration, and sink should be clean and organized

    The items need to be handled:
    A. your samples, solvents, reagents, tips, vials, containers
    B. your wastes: gloves, Kimwipers, cleaning paper
    C. your data, articles, other printed out materials
    D. Facility tools, syringes, solvent, standard, accessories, washing bottles
    E. Logbook, manuals, and other documents

20. Sign out in Log Book
    Turn off lights and lock the door when leaving

DATA PROCESSING AND PRINTING

View Data:
File – Open – Select your folder under C>Hitachi Services> Select and open your data file

Print Data (do the above first, then)
   a. Data – Report-Print -(pages 1-2)-OK
   b. Also select the spectrum (upper screen) OR the Peak data window (bottom screen)
      then, File- Print-OK
Appendix G: Matlab Script for Concentration Profile and Transport Parameter

% ****************************program name: analysis_v9****************************
% modification of analysis_v8
% modified by A. Zak:
% Average value of intensity (concentration) is calculated for each column
% 95% confidence intervals added concentration profiles
% July 28, 2015;
cle;
clear;

% *********************input parameter values for experiment*********************
time_h=24; % enter time in hours
[data]=xlsread('D:s10g8et25d71815c.xls'); %input intensity data,
autoflur=32; % average autofluorescence value for this beam
specimen='Loaded (30 uM): Concentration Curve - Sample 1';
%conc_o=0; initial concentration (after subtracting autofluorescence)
is zero
%**************Variable definitions****************************
% eta       array of dimensionless distance, with values between 0 and 1
% n_cols    number of columns in intensity matrix (i.e. number of pixels
% L         total length of the tissue, in mm
% distance_pixels(i) array containing pixel number
% distance(i) 1D array containing distance, in mm
% n_rows    number of rows of data in each quadrant
% data(i,j) array containing original intensity data, to be modified by
% subtracting autofluorescence
% data_quadrant(i,j) array containing eachquadrant of normalized intensity data
%diff1(i)   array containing all 2500 initial guesses for diffusivity, mm2/s
%diff      single diffusivity value, mm2/s
%diff_table(2600,2) 1st column contains diffusivity guesses (mm2/s);
% 2nd column contains sum of square of errors using guessed diffusivity
%conc_1    boundary condition, C1, intensity at exposed end
%conc_o    initial condition, Co, specified =0 (after subtracting autofluorescence)
is
% subtracted)
%theta(i)   dimensionless concentration=(C-Co)/(C1-Co)
%thetaprime(i)dimensionless concentration=(C1-C)/(C1-Co)
%conc(i)    1D array, concentration of solute, in units of intensity
%sumsq     sum of the square of errors
%MinSSE     minimum of the sum of square of errors

quadrant_name={'All quadrant data'};

%--------------------------****Preliminary Calculations and Functions *******------------%

time=time_h*3600;  % convert time to seconds

%convert the y axis data from pixels to mm
n_cols=length(data(1,:));
distance_pixels=linspace(1,n_cols,n_cols);
L=n_cols*.000812*20;
distance=distance_pixels.*.000812*20;
fprintf('Length of given tissue is %f mm \n \n',L)

%calculate dimensionless distance variable
eta=distance./L;

% find number of rows of data
n_rows=length(data(:,1));
n_rows=fix(n_rows);

%subtract off autoflur value from the entire matrix
data=data-autoflur;

%replace negative intensities with zero-values
%for i=1:n_rows  
  %for j=1:n_cols
    % if (data(i,j)<0)
      % data(i,j)=0;
    %end
  %end
%end

%--------------------------****curve fitting using least squares method****------

%create a table of various diffusivities (2600) from 1e-4 to 1E-11 ranging
%with equal expeditial increasing values between each decimal
  element1=linspace(1E-4, 1E-5, 100);
  element2=linspace(1E-5, 1E-6, 100);
  element3=linspace(1E-6, 1E-7, 200);
  element4=linspace(1E-7, 1E-8, 400);
  element5=linspace(1E-8, 1E-9, 600);
  element6=linspace(1E-9, 1E-10,600);
  element7=linspace(1E-10, 1E-11,600);

%Assign 2600 initial guesses to 1D array diff1
  diff1=[element1 element2 element3 element4 element5 element6
          element7];  %mm2/s

  difftable = zeros(2600,2);
%set boundary condition as average of first 5 intensity values for each quadrant

conc_1=0.;
for i=1:n_rows
    for j=1:5
        conc_1=conc_1+data(i,j);
    end
end
conc_1=conc_1/(5*n_rows);

%intensity profile normalized to concentration profile
tryme=data/conc_1;

%average concentration determined for each column
avg=mean(tryme);
sdev=std(tryme);

%95% confidence intervals added for concentration profile
upper=avg+1.96*sdev/sqrt(n_rows);
lower=avg-1.96*sdev/sqrt(n_rows);

%start loop for iteration of diffusivities, calculating concentration profile for each diffusivity
for k = 1:1:2600
    diff=diff1(k); %mm2/s, assign current diffusivity from array to simple variable
difftable(k,1) = diff; % 1st column of difftable is diffusivity guess

%actual function we are fitting to
thetaprime = 0;
tau=((time.*diff)./L^2); %tau is dimensionless time;
for n=[0:1:100] % infinite series equation to calculate dimensionless concentration profile
    arg1=(2*(-1)^n)/((n+.5)*pi);
c=cos((n+.5)*pi*(eta));
e=exp((-n+.5)^2*(pi^2)*tau);
    thetaterm = arg1*c*e;
    thetaprime=thetaterm+thetaprime;
end
theta2=1-thetaprime;
conc2=conc_l*theta2;
conc2=fliplr(conc2); %change concentrations such that high concentration is at eta=0
theta2=fliplr(theta2);

%calculate the sum of all the squares and place in a table
sumsq = 0;
for j = 1:n_cols
    for i = 1:n_rows
        sumsq = sumsq + ( (conc2(j)-data(i,j))^2 );
    end
end
difftable(k,2) = sumsq/(n_cols+n_rows);
end %k, end iterations of 2600 different diffusivities

% Find Minimum SSE (sum of square of errors)
[MinSSE MinDifIndex] = min(difftable(:,2));

% Rerun model using best fit diffusivity
diff = difftable(MinDifIndex, 1);
tau=((time.*diff)./L^2); %tau is dimensionless time;
thetaprime = 0;
for n=[0:1:100]
    arg1=(2*(-1)^n)/((n+.5)*pi);
c=cos((n+.5)*pi*(eta));
e=exp((-((n+.5)^2)*(pi^2)*tau));
    thetaterm = arg1*c*e;
    thetaprime=thetaterm+thetaprime;
end
theta=1-thetaprime;
conc=conc_1*theta;
conc=fliplr(conc); %flip concentration profile left to right
diff_cm=diff/100; % convert diffusivity to cm2/s for output of value
theta=fliplr(theta);
%Conc is the important variable of insterest that is plotted vs.
distance
fprintf('The best fit diff coeff. is %E cm2/s \n', diff_cm);
fprintf('The normalized SSE is %f \n \n', MinSSE);

%------------------------***Plot functions***------------------------
%figure(s);
plot(distance,theta,'g-',distance,mean(tryme),'b-', distance,
upper, 'r.', distance, lower, 'r.','LineWidth',4 )
axis([0,1.8,-0.1,1])
grid on
xlabel('Distance (mm)')
ylabel('Theta')