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12-19-2018

### Research timeline for exploring methods determining mechanical properties of the mouse anterior cruciate ligament

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#### Recommended Citation

Bram, Zachary and Lake, Spencer, "Research timeline for exploring methods determining mechanical properties of the mouse anterior cruciate ligament" (2018). *Mechanical Engineering and Materials Science Independent Study*. 79.

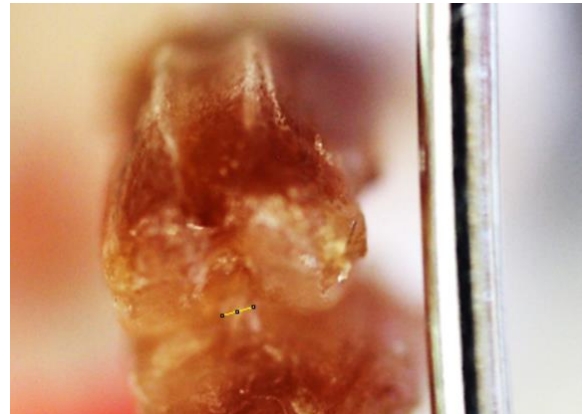
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## Timeline of Fall 2018 research

This semester consisted of working on my research project determining mechanical properties of the mouse anterior cruciate ligament (ACL) in the Spencer Lake lab. Knowing the mechanical properties such as yield strength and stiffness of the ACL would be the first step in designing a replacement ligament using tissue engineering. The first week of the semester began in late August picking up where I left off from the summer using micro computed tomography (uCT) scanning to determine the cross-sectional area (CSA) of the ACL. CSA is a dimension necessary for determining yield stress of the ligament during load-to-failure tests hence of high interest. On the medical campus I was aided by the imaging tech Daniel Lieb using the facility's uCT machine along with his custom designed MATLAB code. Dan's program allows one to go through a uCT scan, create a contour to map the ACL where it is seen, and export the stack as a 3D image. For my ACL bundle, the width of the model would be measured as the thickness of the ACL. While this method worked for various tissue samples and bones, it could not successfully determine the ACL thickness. The code that was created ran into a bug that not even Dan could figure out. The issue lied with the push button "execute analysis" which was needed to be used in order to properly create a 3D model. Regardless of how well defined the ACL was mapped, an error was seen on all school computers on both the Danforth and medical campuses stopping the analysis from being done. Dan left WashU for another job prior to the start of the Fall semester, and the new imaging tech had no idea where to start for trouble shooting. I was recommended using the uCT computer for performing the analysis process instead of using MATLAB, but there was no guarantee it would work any better.

Exploring other methods of determining CSA, I tested using our lab's macro lens camera to take a picture holding a small wire next to the bundle to act as a scale bar. The width of the ACL was measured using ImageJ determining the width of the wire in pixels followed by the width of the ACL (figure 1). The wire was measured to be 0.88mm in diameter using a dial caliper, so the ACL dimension was converted from pixels to millimeters given this. While not as accurate as using uCT, this method proved to be enough for determining an approximation for CSA to continue moving forward.

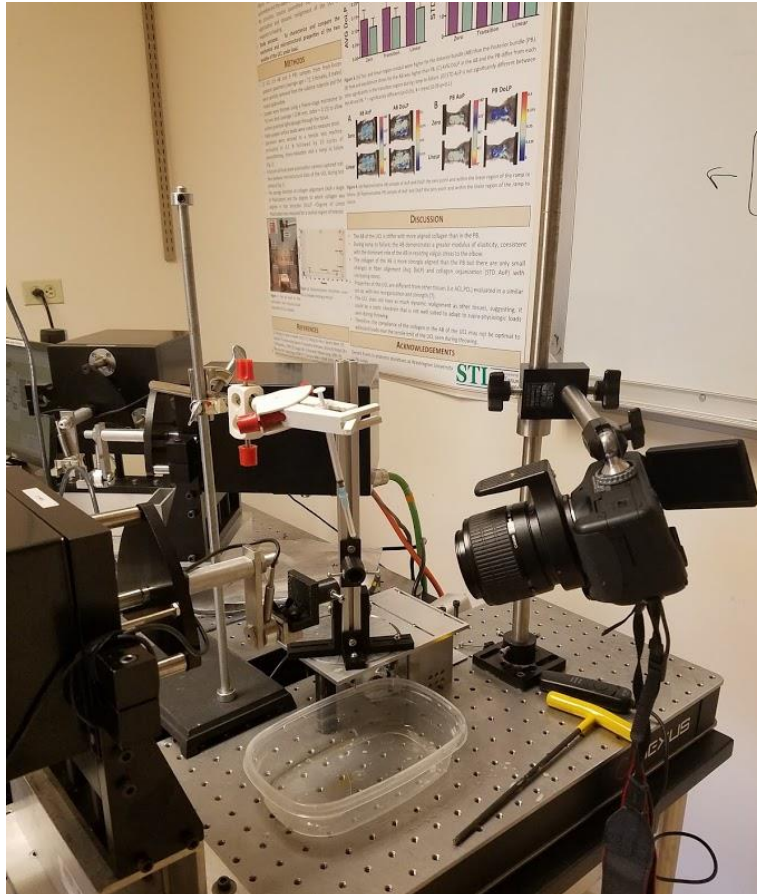


**Figure 1: Measuring mouse ACL width using ImageJ**

My focus in September was to make the process of loading to failure the mouse ACL bundle successful and repeatable. The mouse ACL is approximately 1mm long and less than ½mm wide. To determine its yield force and how long it can deform before tearing, the ACL must be the only connective tissue between the femur and tibia. Preparing the bundle of the femur-ACL-tibia for testing, the bundle is dissected off a thawed sample mouse removing all muscle on the femur and tibia before potting the limbs in plastic cylinders using bone cement. The bundle is

then taken to a dissecting scope in the Setton lab to remove all the connective tissue aside from the ACL.

There were many challenges that needed to be overcome to perform a successful mechanical test of the mouse ACL that include mounting the ACL bundle onto the mechanical testing apparatus without tearing the ACL, keeping the tissue hydrated, observing how well the ACL tore during tests, and eliminating all observed flexion during testing. Keeping the ACL hydrated is necessary to mimic an *in vivo* environment (being in the body). Should the ACL dry out, it would be stiffer than normal giving inaccurate mechanical properties. Using supplies around the lab I created a



**Figure 2: Mechanical testing apparatus prior to loading. ACL is mounted with load cell ready to pull the tibia to the left while femur is held in place by a stand. Tissue is hydrated by dripping PBS through a syringe while a camera records live feed of the load-to-failure test.**

drip system out of a lab mixer plate stand, a clamp, a plastic syringe and a 26-gauge needle. This setup would drip PBS onto the ACL at approximately 1 drop a second with the syringe at a certain angle.

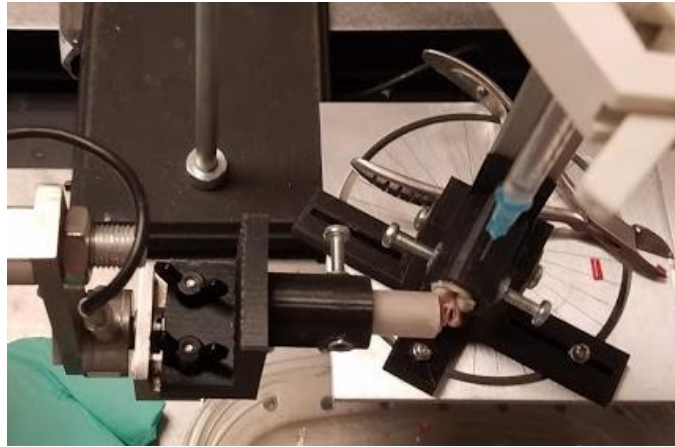
The hardest challenge that I spent the most time troubleshooting was mounting the bundle onto the testing apparatus. Typically, the bundle would tear due to torsion experienced in the palm of my hand, or due to compressing the ACL bundle together when adjusting the load-cell. I tried various ways to work around this thinking to incorporate a support between the femur and tibia pots. This solved the problem for mounting the bundle onto the testing apparatus but created a new problem tearing the ACL when cutting the support. The vibration caused by snipping the support regardless of the material (toothpicks and coffee straws were both tested) would snap the ACL like nothing. Each week during September I would dissect the hind

limbs of mice from the lab freezer but only a few were successfully tested from start to finish obtaining a yield force. Five limbs out of thirteen were successfully tested seeing an average of 3N for the yield force and an average of 2.5mm for the displacement seen at the yield force the first seeing a yield force of 2.85N at a displacement of 2.65mm and the second seeing a yield force. All tests were performed with the ACL at 60 degrees of flexion (figures 2 and 3).

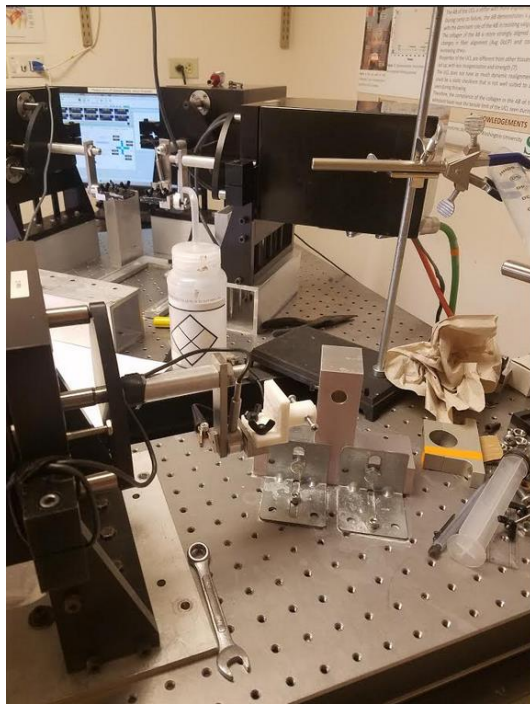
The average ACL dimensions using ImageJ were also recorded from the 5 successful samples averaging 0.326mm for the width and 0.76mm for the length from the ACL insertion to exiting point.

The issue of successful mounting of the mouse ACL bundle continued into the first week of October. Progress was made when the surgical resident, Ryan Hill, returned to the lab from the summer to help troubleshoot this problem and aid in dissecting the mouse knee joints. His help allowed me to focus on the problem of mounting without spending hours performing knee joint dissection to prepare samples for mechanical testing. What ultimately solved the mounting problem was potting the ACL bundle at a 60-degree angle prior to mounting.

Before the bundle was potted, I focused on making sure the femur and tibia were submerged as much as can be without covering the knee joint. With the bundle already at a 60-degree bend, the femur could be inserted into the pot stand and the load cell could be adjusted smoothly without compressing the bundle and tearing the ACL.



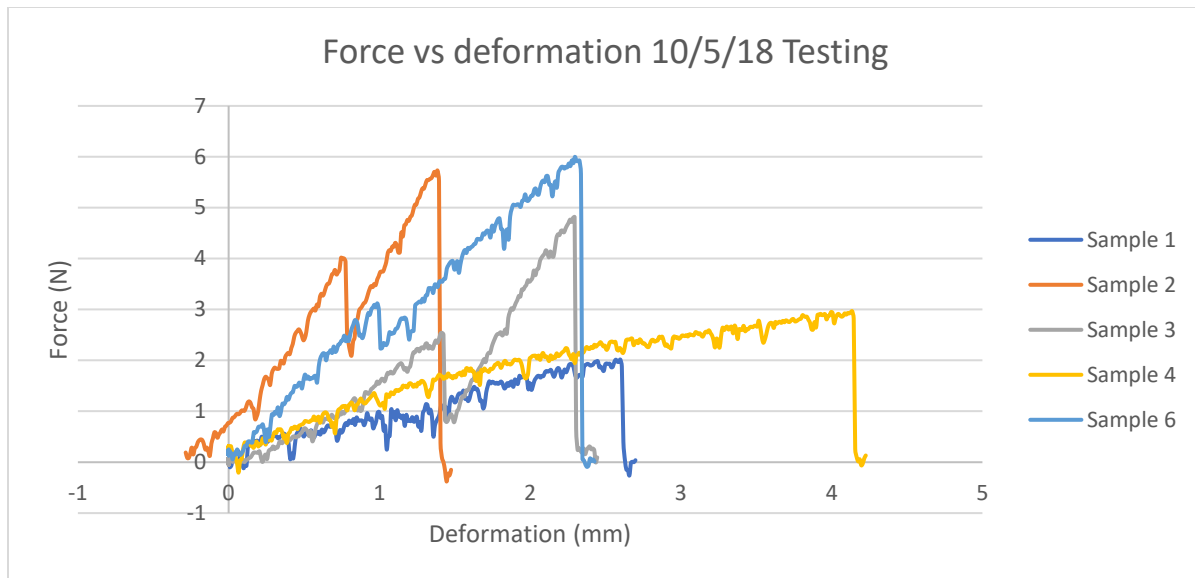
**Figure 3: Mouse ACL bundle close up mounted in mechanical testing apparatus at 60-degree bend**



**Figure 4: Updated mechanical testing apparatus**

With the mounting problem solved, I could move on to investigate removing all minor bending during mechanical loading the other challenges. During mechanical testings performed in September, an observation that was made looking at recorded footage of ACL mechanical tests. It was seen that the entire ACL bundle moved with the load-cell prior to the ACL tearing. This is an issue because to accurately determine strain, the ratio of change in displacement to the initial ACL length, there cannot be any displacement. Looking back at the apparatus, the stand that holds the femur in place was seen to bend in the direction of the load cell with a sufficient amount of force. The design of the stand was previously made with the idea to load an ACL bundle at various angles but at the cost of not being completely secured to the table. I designed a new pot stand shaped as an upside-down T that mounts directly onto the table using L-brackets (figure 3).



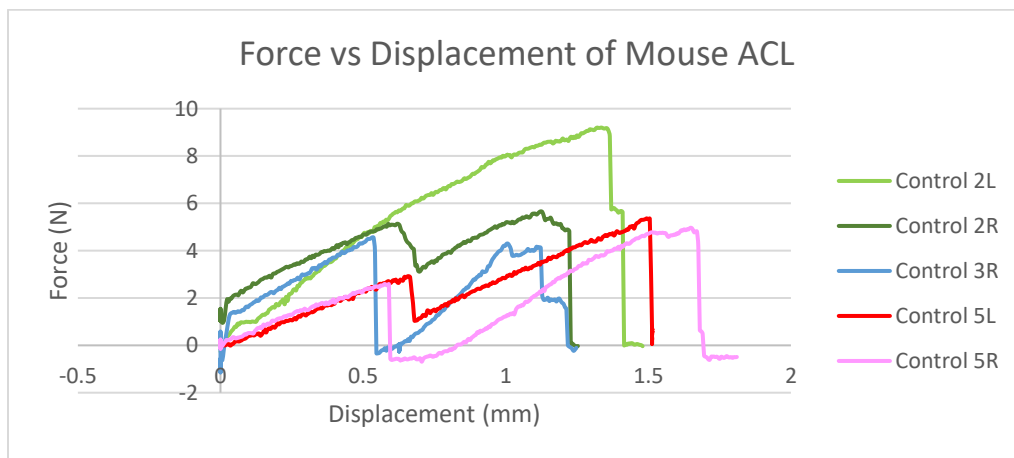


**Graph 1: Mouse ACL load-to-failure testing on practice freezer mice**

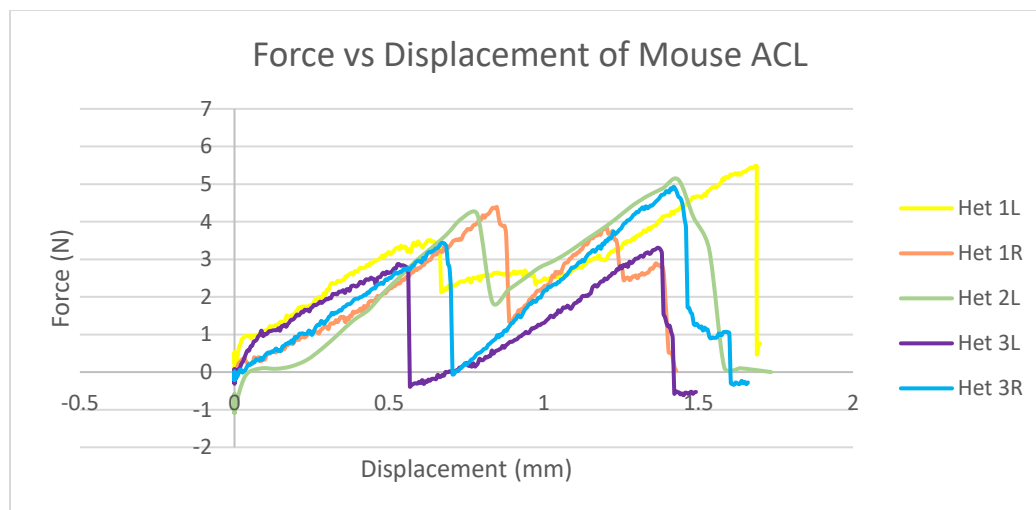
After making all the changes, I was able to successfully test 5 ACL bundles out of 6 samples in one week getting an average of 4.3N yield force with an average deformation of 2.55mm (graph 1). With the challenges solved and mechanical testing repeatability improved significantly, Spencer and I felt ready to test mice from the Farooq lab and try to collect sample data to be used for a journal study and possibly a grant. The Farooq lab is a lab on the medical campus Spencer's lab has been looking to collaborate with to determine how mouse ACL mechanical properties correlates to changes in elastin and collagen content. I received 8 mice from their lab to compare mechanical properties: 5 control wild type and 3 heterozygous periostin types. I prepped all the samples for knee joint dissection having Ryan perform the all the knee joint dissection. Before each mechanical test, I took a picture of the ACL to measure the CSA and exposed length using ImageJ as mentioned earlier. Stiffness and young's modulus were calculated using a custom MATLAB program from one of the Ph.D. students in the lab, Chelsey Dunham. The program calculated the slope of the linear region of a graph for two types of graphs: force-displacement and stress-strain. Stiffness is the slope of the force-displacement curve and young's modulus is the slope of the stress-strain curve. All mouse samples were successfully tested except for the first control mouse's left limb that experienced the femoral condyle remove before the ACL tore. This failure can be due to the larger time gap in between dissecting and mechanical testing being 5 days instead of 1-2 days. The bundle was wrapped in gauze soaked in PBS in the refrigerator but was still rotting, nonetheless. An issue that arose for the first half of testing was the bone cement keeping the femur and tibia still in the plastic potting tubes. During mechanical testing for almost all the control mice, limbs were pulled from the pots and had to be repotted to successfully tear the ACL. This problem was solved after getting a fresh can of hardening bondo, and by applying Loctite to the femur and tibia prior sticking them into the pots filled with bone cement. The Loctite tip was recommended to me by our lab manager saying it was actually common for the bones to slip out of the pots, and that the contact of Loctite-bone cement was

significantly more secure. The data collected was reasonable but underwhelming because there was not a significant difference between the control and heterozygous type mice (graphs 2-5).

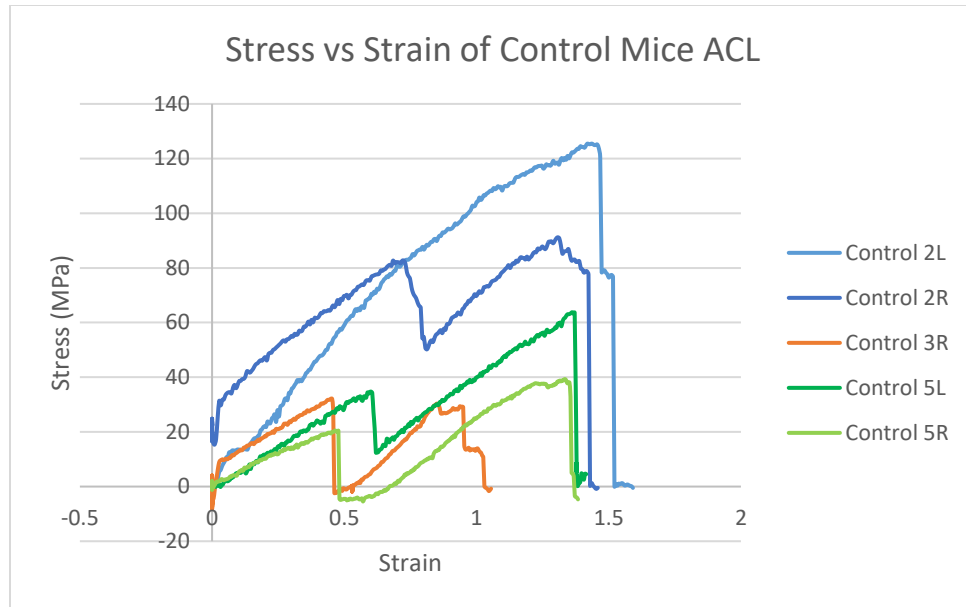
An observation from most of the curves is that there are two peaks instead of one. This is strange because if there was only the ACL connecting the femur and the tibia there should be a single peak. There were also distinct pops heard that indicated when the ACL tore matching the number of peaks seen in the force-displacement curves. Showing Spencer and the rest of the lab researchers my findings there were two suggestions made. The first idea was that the mouse ACL, tiny as it may be, has two bundles and experiences two tears depicted by the two peaks. The second idea was that there is other connective tissue between femur and tibia resulting in two tears depicted by the two peaks in most samples.



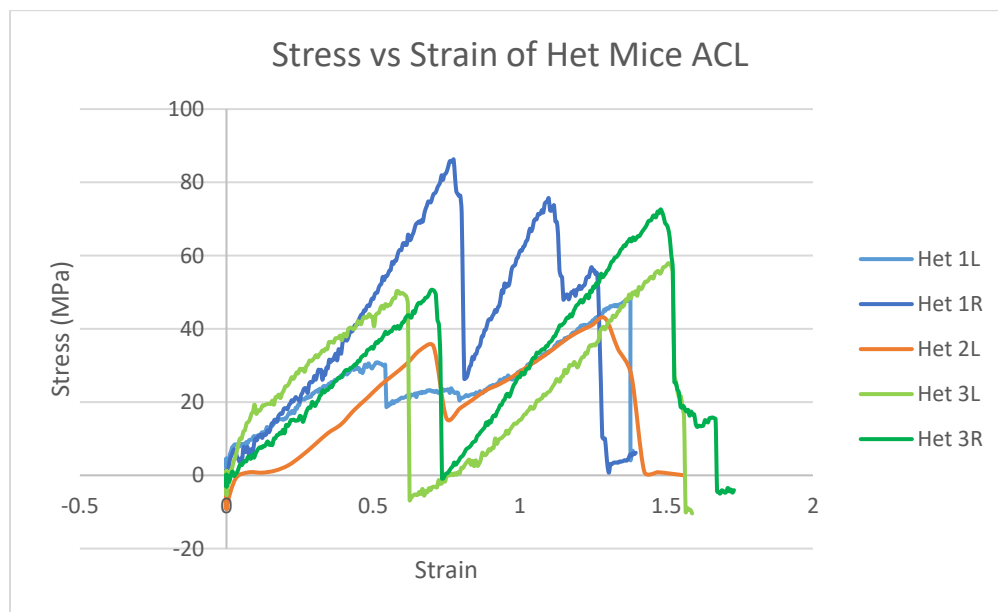
**Graph 2: Force vs displacement of mouse ACL for successfully tested control wild types**



**Graph 3: Force vs displacement of mouse ACL for successfully tested heterozygous periostin types**



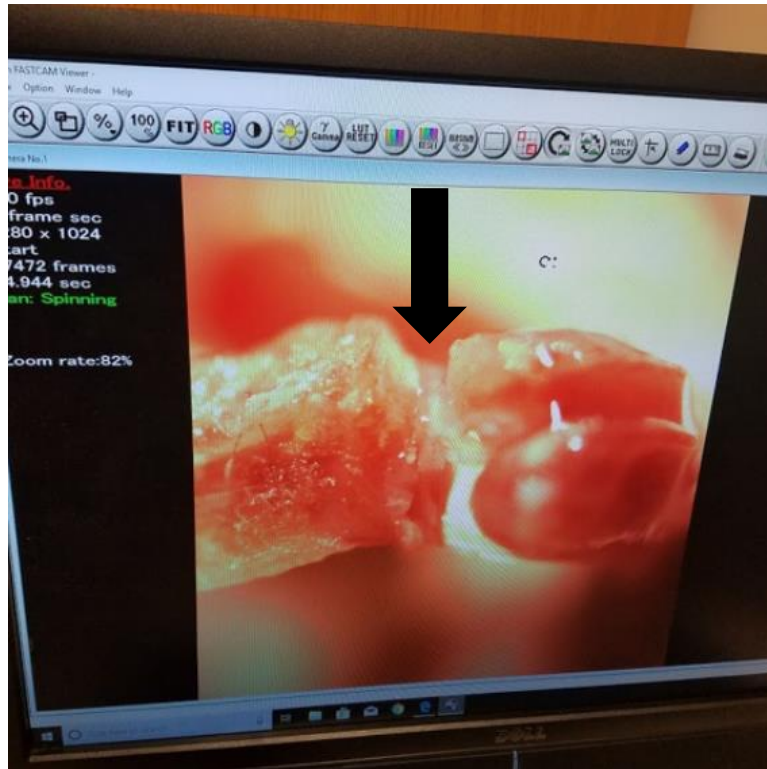
**Graph 4: Stress vs strain of mouse ACL for successfully tested control wild types**



**Graph 5: Stress vs strain of mouse ACL for successfully tested heterozygous periostin types**

By now it was early November and unfortunately, we had missed the deadline for the Farooq lab to apply for the grant this preliminary data would be used for. Moving forward Spencer wanted me to determine whether there is leftover connective tissue in the knee joint aside from the ACL to explain why multiple peaks were seen in the Farooq data. He recommended that I use our lab's high-speed camera to see whether or not the ACL tears in a single snap or in intervals. During limb preparation I made a major discovery when some of the researchers were double checking my knee joint dissection. There was a zoom knob feature on the dissecting scope that was not used by me or Ryan. Using the feature resulted in a notable difference in the gap

between the femoral condyle and the tibia head after removing all connective tissue aside from the ACL (figure 5). This made a difference visually and numerically with the data. All the samples heard and saw one snap on the camera, and there was only one peak for all force-displacement curves. This proved that there was connective tissue remaining prior to most of the Farooq mice tests resulting in multiple pops and peaks observed in the data.



**Figure 5: Mouse ACL bundle shown on the high-speed camera before load-to-failure test (arrow pointing to ACL)**

This has been a summary of all the work done in the Spencer Lake lab for the Fall 2018 semester. While the process has been long and ran into many unexpected challenges, it has been rewarding work. While the semester may be over, I am waiting to hear back from the Farooq lab to see if I can test some elastin knockout mice and compare them to the other two mice types. The data collected before, while not perfect, has accurate yield force and deformation data looking at the second peak from each sample curve. Time provided, I plan to submit an abstract to an upcoming conference.