California Polytechnic State University, San Luis Obispo

A Senior Project

Development and Implementation of Assessment Methods for Tissue-Engineered Blood Vessel Mimics

In Partial Fulfillment
of the Requirements for the Degree
Bachelor of Science

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Abstract

Coronary Artery Disease (CAD), the most prevalent form of heart disease, is the result of clogged or damaged coronary arteries and claims around 380,000 Americans annually. A common treatment for CAD involves placing a stent into the artery in order to open the lumen and support the native tissue—a procedure that drastically reduces patient recovery times in comparison to heart bypass surgery. However, stents do not always interact well with the body and require additions such as surface coatings or drug elution in order for additional biocompatibility. These additions necessitate extensive *in vitro* and *in vivo* testing which are expensive and yield limited data concerning the human physiological response. To address this gap in testing, the Tissue Engineering Lab at Cal Poly San Luis Obispo has developed a protocol to produce blood vessel mimics (BVMs) for the purposes of realistic *in vitro* evaluation of stents and other cardiovascular therapies.

The purpose of this project was to implement two BVM evaluation methods to test the outcomes and repeatability of the BVM protocol: compliance testing and cryosectioning. For compliance testing, a fixture, software, and a protocol was implemented and tested with the goal of obtaining repeatable compliance measurements. For cryosectioning, a protocol was implemented to obtain quality sections and stains for future use by the lab. Through the implementation of the compliance tester, it was found that the computed compliance may vary substantially due to a number of factors and thus a few major improvements were proposed, including purchasing a laser micrometer and a syringe pump. Proposed future work for the cryosectioning includes an in-depth characterization of the factors that contribute to quality sections which may include BVM thickness and BVM fixation lengths. Overall, this project increased BVM evaluation capacity in the Cal Poly Tissue Engineering Lab by providing additional methods to ensure manufacturing repeatability.

1 Introduction

Heart disease is responsible for 1 in every 4 deaths in America each year and is the leading cause of death in both men and women [1]. Coronary Artery Disease (CAD) is the most prevalent type of heart disease, claiming around 380,000 Americans annually [1]. Thus, a large need for treatment of CAD has led to the development of treatments and technologies such as stents, heart bypass surgeries, and tissue engineered blood vessel replacements. Stents are cylindrical metal scaffolds that are placed into an artery typically via balloon catheter in order to open a damaged or clogged artery. Stents are typically the preferred treatment method because their implantation procedure is minimally invasive [13], whereas both bypass surgery and artery replacement require surgical access to the heart.

Due to the dramatic differences in hospital discharge times between the stenting and artery replacement surgeries—1 day for stenting and 6 to 8 weeks for heart surgery [4]—researchers are developing ways to give stents additional physiological benefits such as drug elution or increased biocompatibility. However, testing such additions requires both extensive *in vitro* tests that, although relatively cheap, yield limited physiological data, and expensive and harmful animal testing that do not fully mimic true physiologic conditions. To address this gap in testing techniques, the Tissue Engineering Lab at Cal Poly San Luis Obispo—directed by Dr. Kristen Cardinal—has developed a protocol to produce blood vessel mimics (BVMs) for the purpose of evaluating stents with human cells in a simulated physiologic environment. In recent years, the lab has worked on developing a reproducible BVM protocol with relative success, which has necessitated additional analysis techniques to validate BVM reproducibility [2][3].

The BVM manufacturing process can be split up into two major components: first, the scaffolds are created by electrospinning Poly (D,L-Lactide-Co-Glycolide)—PLGA—into cylindrical tubes [3], and second, cells are sodded onto the scaffold and then cultured for 1 to 3 days within a bioreactor under flow [2]. Thus, the analysis methods to determine reproducibility must address both manufacturing methods. To analyze the scaffold reproducibility, tensile testing and fiber diameter analysis have been performed, and to analyze cell sodding reproducibility, fluorescent en-face imaging, SEM imaging, histology, and gene expression have been used. The purpose of this project was to implement two new, but previously researched and developed, analysis

techniques for use by the Cal Poly Tissue Engineering Lab: compliance testing for PLGA scaffolds and cryosectioned histological analysis for cell-sodded constructs.

2 Compliance Testing

To evaluate electrospun PLGA scaffolds, it is necessary to measure how the scaffold will behave as a blood vessel mimic (BVM). There are many ways to do so, such as testing mechanical properties that gauge the strength or elasticity of a tissue engineered scaffold, however, these assessments must damage the sample prior to testing and do not take into account the effect of physiologic conditions. Since blood vessels are tubular in nature and must stretch and contract in response to cyclic blood pressures, in order to evaluate how accurately BVMs will mimic native blood vessels, it is necessary to measure compliance: the radial deformation under pressure.

2.1 Background

There are many different ways to describe compliance, however ISO 7198 establishes a standardized method [5]. The most basic mathematical description of compliance within a vessel is change in volume over change in pressure, as shown in Equation 1 [6].

$$C = \frac{\Delta V}{\Delta P} \qquad \text{Eq. 1}$$

However valid, Equation 1 is most commonly used for sac-like structures such as the bladder that fill with fluid. Since blood vessels do not store blood—blood simply passes through at different pressures—compliance must be described in different, measurable terms: usually in terms of radius or diameter change [7]. However, when substituting change in volume with change in diameter, a percent change in diameter must be used since, for example, a 5 mm change in diameter can represent very different changes in volume if the original diameter was 2 cm versus 10 cm. This leads to the compliance equation outlined in ISO 7198 (Equation 2) [5].

$$\%\, Compliance = \frac{\left(\frac{D_{P_2}^2 - D_{P_1}^2}{D_{P_1}^2}\right)}{P_2 - P_1} \cong \frac{\left(\frac{D_{P_2} - D_{P_1}}{D_{P_1}}\right)}{P_2 - P_1} \; for\ small\ \Delta D \qquad \text{Eq. 2}$$

Equation 2 depicts the mathematical model for physiologic compliance. By relating the percent change in diameter to the overall change in pressure, Equation 2 measures how vessels respond to variations in physiologic pressures. Additionally, since this equation is the established method for calculating compliance, it allows researchers to be unified in their data collection methods. Indeed, compliances of certain arteries within the human body are on their way to being well-categorized [8], which will give engineers the necessary information to develop physiologically similar BVMs.

With an established equation to calculate compliance, it became necessary to develop methods to apply pressure, record pressure, and record diameter change on the PLGA scaffolds used in the Cal Poly Tissue Engineering Lab. To this end, A. Rowley and S. Tipton developed a compliance test fixture that utilizes a fluid-filled balloon catheter to apply internal pressure to the scaffolds, a pressure transducer to record the applied pressures, a microscope/camera system to record the diameter change, and a custom Matlab program to extract the diameter change from the recorded video [9]. The completed fixture they developed can be seen below in Figure 1.

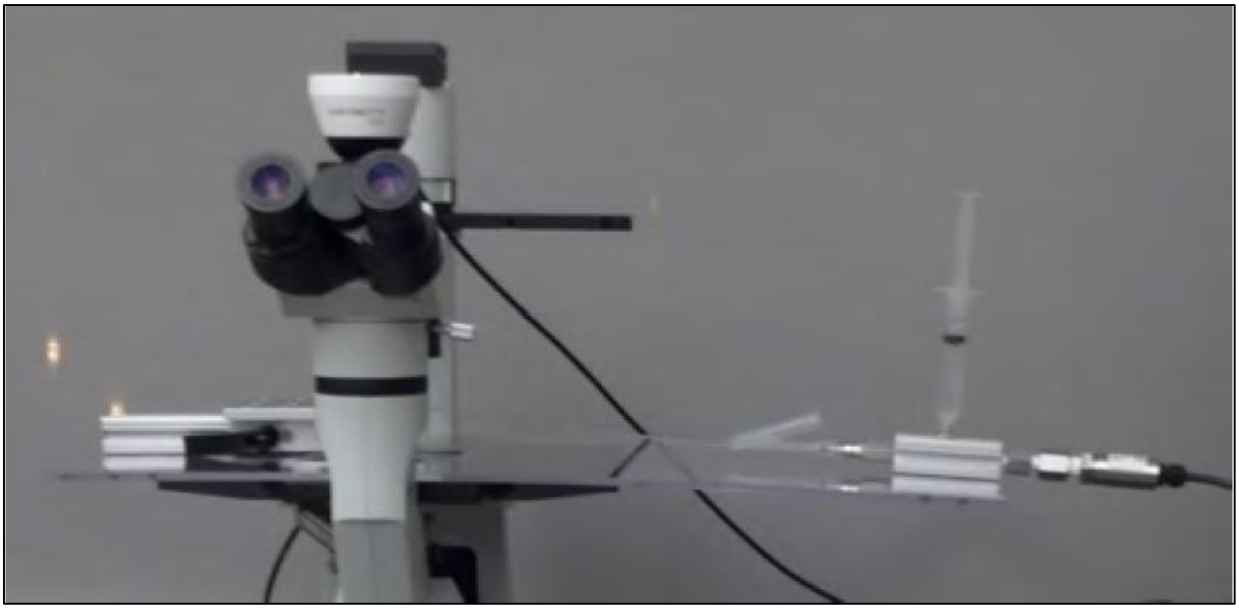


Figure 1: The compliance fixture developed for the Cal Poly Tissue Engineering Lab by Aaron Rowley and Shane Tipton.

As shown in Figure 1, this fixture was composed of an acrylic sheet with a viewing hole cut-out that the stock 80/20 pieces were mounted to [9]. The rightmost piece served as the pressure conduit to transfer the pressure from the syringe to both the balloon catheter and pressure transducer [9]. The left piece was composed of a rail and slider system that served to affix and position the catheter [9].

The software to analyze the video, developed in Matlab Simulink, calculates the percent diameter change by measuring the initial and maximal diameters in number of pixels [9]. The program does this by converting each frame into binary (black and white), counting the number of black pixels (representing the scaffold), and dividing by the resolution of the width [9]. To record the pressure change, an Omega DAQ and Omega pressure transducer were used along with associated software to output an excel file of the voltage readings over the course of the test.

Although the above fixture was developed as part of a senior project, it was never implemented for use. Therefore, the purpose of this aspect of the current project was to implement the developed compliance fixture for use by both the Tissue Engineering Lab and by the Biomaterials Laboratory (BMED 420), along with making any improvements to the fixture and software as necessary.

2.2 Fixture Improvements

The first aspect of the project was to reconstruct the disassembled fixture and make improvements wherever necessary to develop a robust compliance tester. This led to a number of improvements: a sturdier acrylic base, a clamp fixture for the catheter, a rearrangement of components affixed to the base, and a new pressure transducer. The design process and final product can be found below in Sections 2.2.1 and 2.2.2, respectively.

2.2.1 Design Process

Upon receipt of the disassembled fixture, the original acrylic base had disappeared. Therefore the first improvement that had to be made was manufacturing a new base. The original base was a single piece of acrylic that flexed considerably as it hung over the end of the microscope stage (see Figure 1). Additionally, the 80/20 components were affixed to the base using thru-holes,

nuts, and bolts. These two features of the old base prompted the use of thicker (0.5”) acrylic with an additional leg to match the height of the stage and provide stability to the fixture. The thicker acrylic addressed the above issues by first allowing the bolts to thread directly into the base, thus minimizing the number of hardware components, and by second providing more rigidity to the structure, in addition to the leg. The design for the acrylic base can be seen in Figure 2 and detailed drawings can be found in Appendix C.

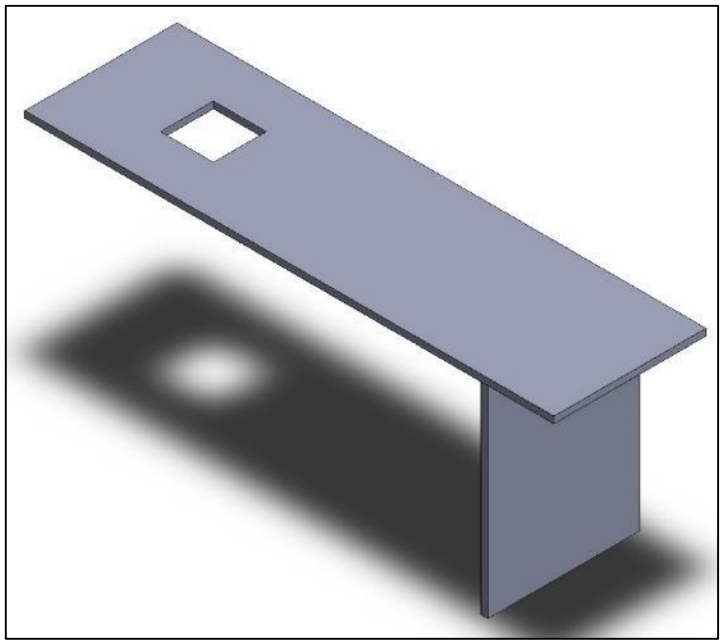


Figure 2: Design of acrylic base to mount the compliance fixture components

Once the base was designed, and construction focused on the mounting of the 80/20 components, the second and third improvements became obvious. With the original design, the open end of the catheter was attached to the middle of an 80/20 bar whereas the balloon-side of the catheter was taped to the top of a slider on top of an 80/20 bar as shown in Figure 3.

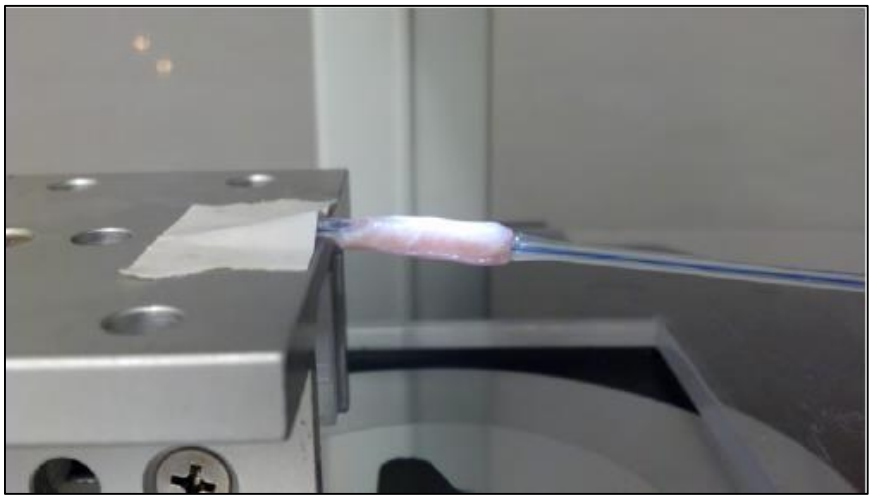


Figure 3: The original method of affixing the balloon catheter to the sliding stage.

As can be seen in Figure 3, the catheter and sample were diagonally centered over the microscope lens in the original design. This could lead to significant measurement errors since the diameter analysis averages the diameter of the scaffold over the entire image: if one end is further from the lens than the other, it will appear smaller. Therefore, to address this error, the first improvement was to rearrange the component orientation in order to level the catheter. Since the syringe and pressure transducer were already connected to the central canal of the pressure conduit 80/20 component, the open, proximal end of the catheter also had to stay centered in the pressure conduit. Therefore, it was necessary to adjust the location of the catheter's distal end to be fixed in the center of the left 80/20 component. Because the distal end must be adjustable in order to compensate for slight differences in catheter length, it was desired to utilize the modified bike wheel clamp A. Rowley and S. Tipton manufactured. However, in order to do so required a reversal of the stage and bar to affix the stage on the base and the bar within the stage. This orientation allows the bar to slide within the stage and is easily mirrored by the pressure conduit. An image of the final 80/20 component's orientations can be seen below in Figure 4.

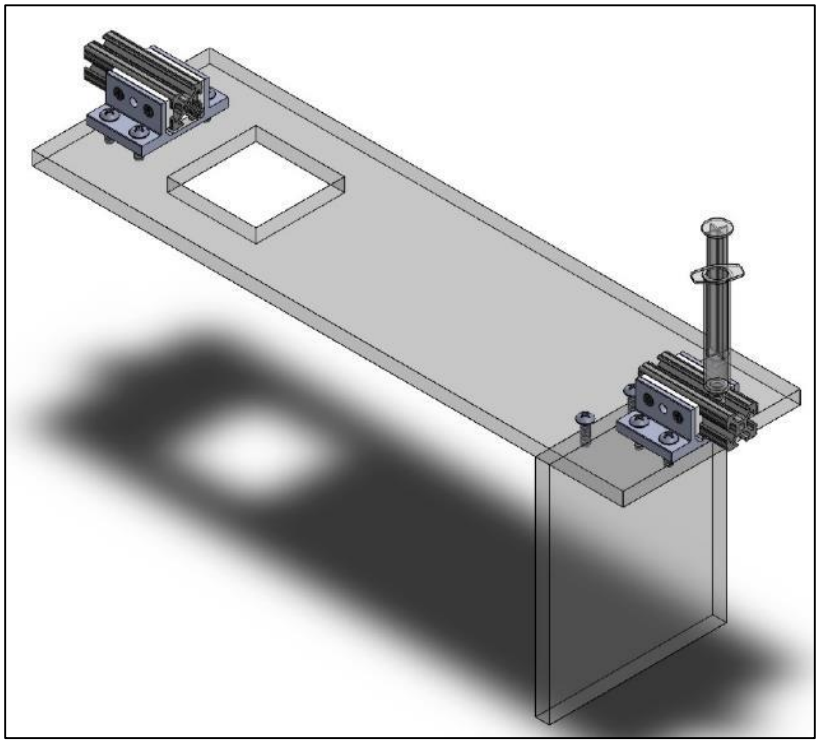


Figure 4: Isometric view of 80/20 components mounted on the acrylic base. Notice the stages are attached to the acrylic while the bars are attached to the stages.

With the component orientations adjusted, the next step was to devise a method for affixing the catheter in the center of the 80/20 bar. The most commonly-used method for affixing samples for testing is via a clamp, therefore a clamp was pursued. Preliminary sketches can be seen below in Figure 5.

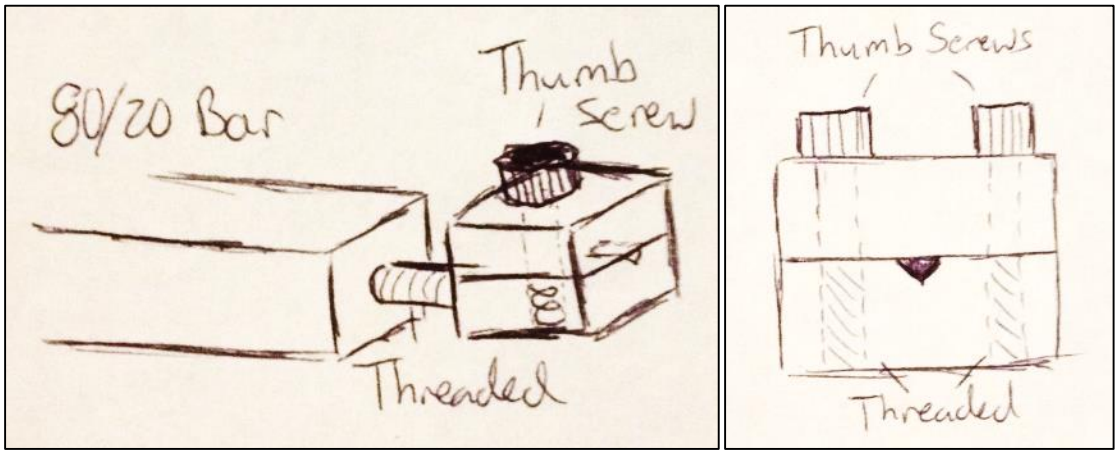


Figure 5: Preliminary catheter clamp sketches. Left, method of attachment with single thumb screw; Right, double thumb screw design.

As shown in Figure 5, the clamp was designed to be two pieces that would tighten around the catheter—placed in the v-slot—using a thumb screw. The entire assembly would then be threaded into the 80/20 bar stock. However, in order to thread into the bar stock, additional features had to be added as can be seen in Figure 6. Additionally, due to minimization of features for ease of manufacturing, it was decided a single thumb screw would be sufficient to clamp the catheter.

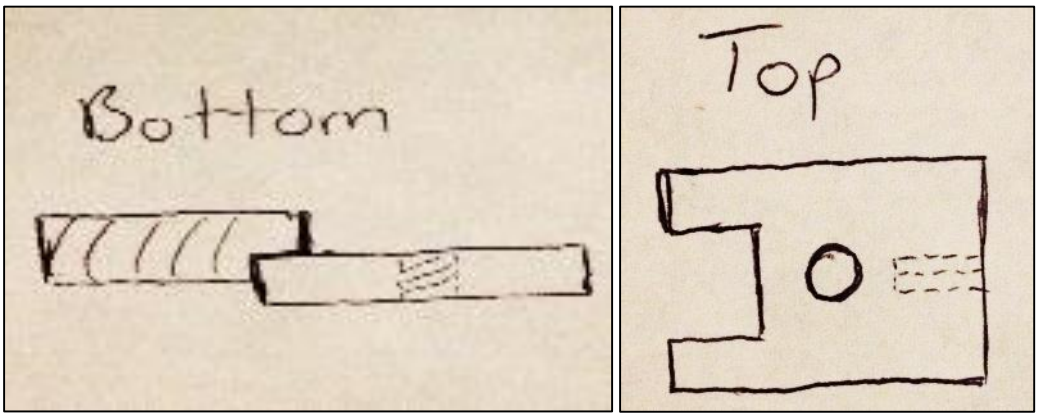


Figure 6: The second iteration of the clamp to include an extension of the bottom plate that would be turned into a threaded rod.

With suggestion by Dave Laiho, the above design was altered to utilize a 1/4-20 setscrew instead of machining a threaded rod from scratch. The final design can be seen below in Figure 7 and detailed drawings can be found in Appendix C.

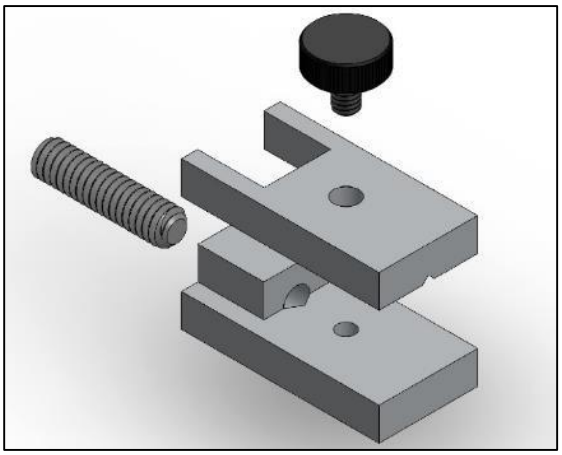


Figure 7: Exploded view of the clamp design.

2.2.2 Product Realization and Final Product

The acrylic base was drawn and dimensioned in AutoCAD, then cut using the Versa Benchtop Laser Cutters in the IME labs. To mount the 80/20 components, holes were drilled and tapped using the Mustang ’60 machine shop. The completed base and mounted components can be seen below in Figure 8.

Figure 8: Completed acrylic base and mounted components

The clamp was manufactured on a mill in the Hangar machine shop with the help of Loren Sunding, then drilled and tapped in the Mustang ’60 machine shop. The design did not change from the manufacturing process and turned out to fit the fixture and catheter perfectly. The final product can be seen below in Figure 9.

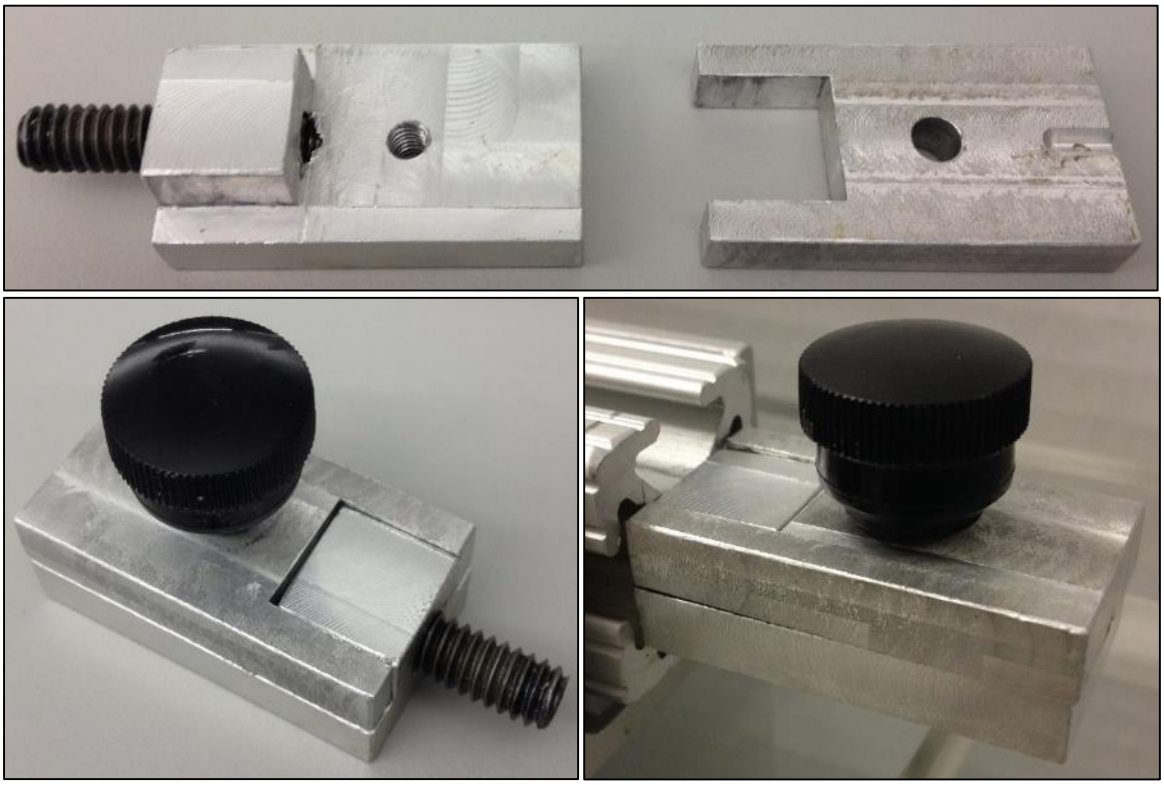


Figure 9: Completed catheter clamp. Top, disassembled bottom and top of the clamp; Bottom-left, assembled clamp; Bottom-right, clamp screwed into 80/20 bar.

With the fixture built and constructed, and using the original software created by A. Rowley and S. Tipton, the fixture and adjusted protocol were tested out for a quarter in the Biomaterials Laboratory (BMED 420). From this testing, it became obvious that a limiting factor of the fixture was the pressure transducer as the testing could not proceed past 15 psi, which was often necessary in order to observe any changes in diameter of the tubing samples used. Therefore a new transducer was purchased that measures up to 50 psi (see Appendix B for the datasheet). However, if lower pressure testing is desired in the future, the 15 psi transducer can be easily swapped back into the fixture using the threads and step-downs used in the original fixture [9].

2.3 Software Improvements

Once the fixture was built and working correctly, it became obvious that the final improvement to make the testing apparatus useable for research, was an update to the software—as it was extremely slow and had numerous operator steps that could be handled automatically. The original software protocol can be found in A. Rowley and S. Tipton's report [9].

2.3.1 Design and Implementation

The original software was developed in Matlab Simulink, a "drag-and-drop" type of programming. Thus, it was unclear exactly how efficiently the code operated, especially that of the video analysis program, because there was not any written code. Therefore the first step in developing the new software was simply rewriting the Simulink analysis program in Matlab code to optimize its efficiency. This was first accomplished by writing custom binary conversion code to combine the loops that perform binary conversion and black pixel counting. By combining the two loops into one, the program only had to perform half of the work, and indeed the runtime to analyze a video decreased by roughly half (see Section 2.3.2). However, with further research into Matlab image analysis capabilities, a built-in function was found that would calculate the area of white in a binary image. Although the compliance program needs the black area, a simple subtraction from the total image area yields this information. Thus, with this new function (bwarea), the image analysis program was rewritten to use it and the built-in function for binary conversion. When tested against the first version of the new software, the built-in functions decreased the runtime more than six-fold (see Section 2.3.2 for full results). Thus, with the built-in functions, the analysis program was completed (see Appendix D for the final code).

With the analysis program finished, it became clear that an additional benefit of rewriting the code in Matlab was the ability to combine the originally separate video capture and analysis programs into a single, user-friendly package. With the discovery of Matlab GUIs (Graphical User Interfaces), it was decided that the next and final step in the development of the new software was to create a GUI that allows the user all necessary control to both capture and analyze the compliance video. To accomplish this, much research had to be done in figuring out how to capture and save video. The final solution was to read in the video footage from the camera frame-by-frame and then save the collection of frames as a single .avi output (the final

code can be found in Appendix D). With the video capture program created, the final step was to create a GUI with buttons to initiate video capture and video analysis, with a video viewer for the user to see the captured and analyzed video. The final product can be seen in Figure 10 below.

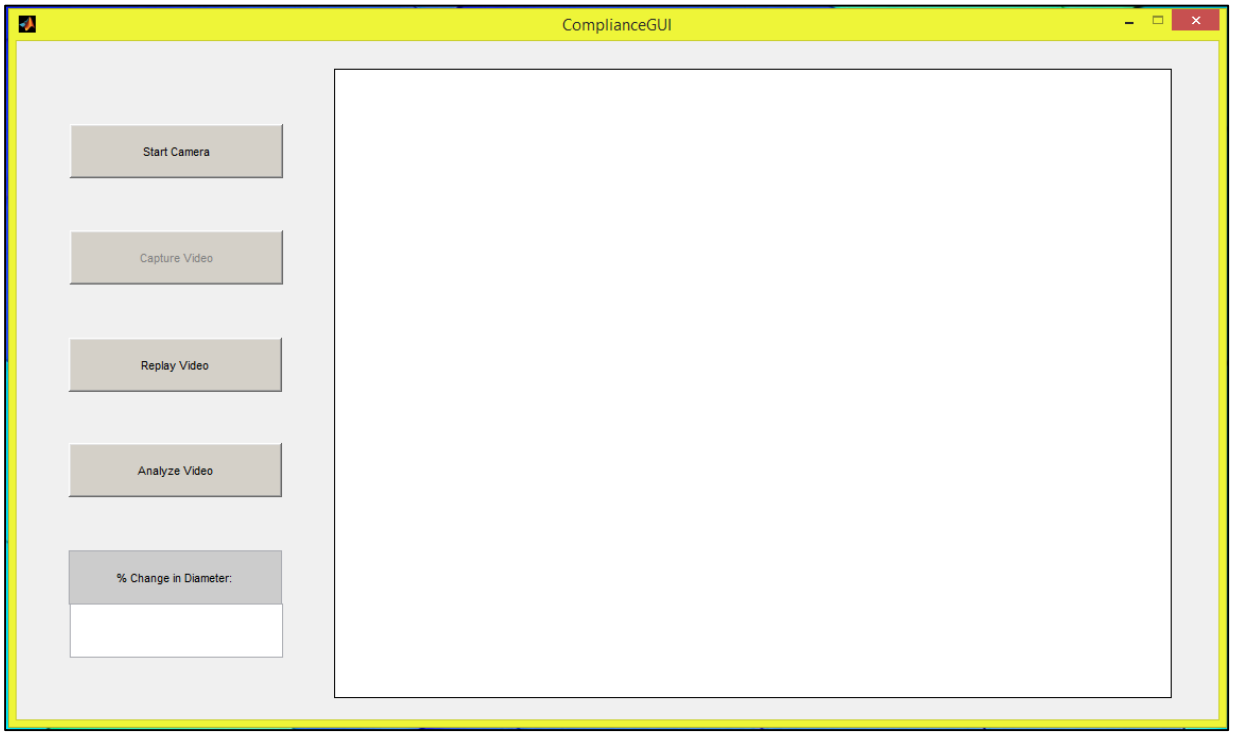


Figure 10: Final compliance tester GUI.

The GUI was designed with the user and protocol in mind and thus contains 4 buttons: “Start Camera”, “Capture Video”, “Replay Video”, and “Analyze Video”. The “Start Camera” button was included to provide the ability to toggle the camera feed on and off to avoid continually heavy draw on the computer’s resources. The “Capture Video” button was included to provide the users the ability to see the video feed without actually recording for ease of positioning the scaffold. Finally, the “Replay Video” button was included so that the user could verify the video they recorded was adequate. The “Analyze Video” button was included for obvious reasons. For additional user-friendliness, the buttons were created to activate when it would be appropriate to press them, and deactivate when it would be inappropriate. For example, while recording video, the “Start Camera”, “Replay Video”, and “Analyze Video” buttons are deactivated, leaving only the “End Capture” to be displayed on the active “Capture Video” button. This functionality is also a safe-guard against errors that could arise through misuse.

2.3.2 Testing

With the new software developed, the next step was to evaluate its performance. This was done in three phases: analysis runtime and accuracy testing, individual button functionality, and user testing.

Analysis runtime and accuracy testing was the first round of testing performed to evaluate the rewritten analysis software's performance. This testing comprised of running the three versions—the original Simulink program, the custom binary and black area calculation program, and the final program that utilized built-in Matlab functions—against two pre-recorded videos. This test served to observe the differences in runtime between the three versions while verifying the calculated percent diameter change remained constant. The results of the test can be seen below in Table I.

Table I: Video Analysis Runtime and Accuracy Testing Results.

Video	Program	Runtime (mm:ss)	% Diameter Change
1	Original Simulink	00:39.35	14.21
	1st Version Matlab	00:19.28	13.81
	2nd Version Matlab	00:03.31	13.82
2	Original Simulink	01:02.02	81.93
	1st Version Matlab	00:32.69	79.94
	2nd Version Matlab	00:05.06	79.98

As shown, each version was faster than the previous, and the final version ended up being twelve times faster than the original Simulink program. Additionally, each program outputted roughly equivalent values for the percent diameter change suggesting that the percent diameter change was being calculated correctly.

After testing the analysis software and developing the GUI, some simple pass/fail criteria were evaluated to ensure GUI functionality. The results of these tests can be found below in Table II.

Table II: GUI Evaluation Criteria.

Criteria	Pass/Fail
Does “Start Camera” start the video feed in the viewer?	Pass
Does the text change to “Stop Camera” once video feed is started?	Pass
Does the “Capture Video” button become activated once the video feed is started?	Pass
Does pressing “Stop Camera” end the video feed?	Pass
Does pressing the “Capture Video” button deactivate the other buttons and change the text to “End Capture”?	Pass
Does pressing “End Capture” prompt the user to save the video?	Pass
Is the saved video able to be played by video player software?	Pass
Does pressing “Replay Video” prompt the user to select a video file?	Pass
Does the selected video play in the video viewer?	Pass
Does pressing “Analyze Video” prompt the user to select a video file?	Pass
Does the selected video play in the video viewer in binary format?	Pass
Does the percent diameter change print out to the “% Change in Diameter” box?	Pass
Does the same video produce the same percent diameter change each time it is selected?	Pass

Finally, with the GUI evaluated and determined functional, the last evaluation step was to test the new software with users on the compliance tester computer. This was done by implementing the new software in the BMED 420 lab and updating the lab protocol accordingly (see Appendix E for full protocol). This testing highlighted a crucial limitation of the software since it would experience a fatal error on the fixture’s laptop with less computing power than the personal computer used to develop the GUI. This result suggested that either a more capable laptop would need to be purchased or the software needed to be updated to work on the less-capable fixture laptop. Since a new laptop was the more expensive option, the software was first edited to see if a GUI could be developed to work on the fixture computer. The first attempt at revising the program involved searching for documentation on the fatal error, however the search was fruitless. The next step was to attempt to fully understand each line of auto-generated code created by the Matlab GUI creator, GUIDE, in order to identify where the error could arise. While performing this step, it became clear that much of the auto-generated code was superfluous, which prompted a complete re-write of the code. This final step involved recreating the GUI with explicit Matlab code so that the purpose of every line would be known and no superfluous code would be included. The rewritten program finally solved the fatal error issue and allowed for fast and user-friendly recording and analysis of compliance video. See Appendix D for the full GUI code.

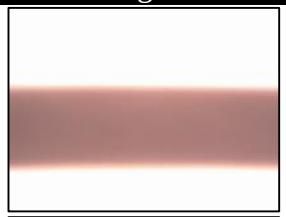
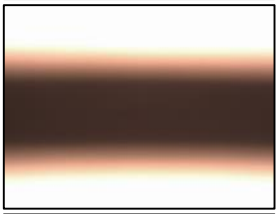
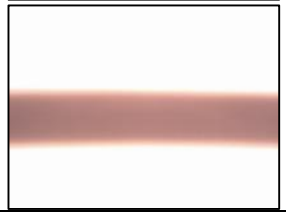
2.4 Compliance Tester Characterization

The final step in implementing the compliance tester was characterizing the effects of the various parameters on the calculated percent compliance; namely, the factors associated with the video capture such as lighting and focus on the microscope. This characterization served to understand the errors associated with the current diameter measurement technique.

2.4.1 Methods

The compliance of a single piece of latex rubber tubing, with 1/32" wall thickness and unknown compliance, was evaluated three times for each of the 9 combinations of two variables: brightness of low, medium, and bright, and focus (image size) of 1/4, 1/3, and 1/2 of the video viewer window. Examples of the 9 combinations can be seen in Table III. Only one tubing sample was used in order to eliminate variation due to a difference in tubing samples, and since the sample was never stressed passed the elastic threshold, the true compliance would not change over time. The protocol used can be found in Appendix E. The trials started at max image size, evaluating all three light intensities, then the size decreased and the three light intensities were repeated, and this process was repeated a third time. During testing, one of the 1/2, light trials' pressure files was overwritten, so two additional trials were run with a different piece of tubing.

Table III: Array of representative images of the 9 trials.

Size	Dark	Normal	Light
1/2 of Video			
1/3 of Video			
1/4 of Video			

A one-way unstacked ANOVA determined statistical significance while Tukey's Method determined which combinations were different. A main effects plot determined which conditions had the largest effects.

2.4.2 Results

To evaluate the effects of image size and brightness on measured compliance, nine combinations of three image sizes and light intensities were evaluated three times each. The results can be seen below in Table IV and Figure 11, the raw data can be found in Appendix H.

Table IV: Measured Compliances for Compliance Tester Characterization (units: %/100 mmHg).

Size	Dark	Normal	Light
1/2 of Video	1.19 ± 0.07	1.37 ± 0.06	2.25 ± 0.26
1/3 of Video	1.48 ± 0.17	1.85 ± 0.13	2.29 ± 0.30
1/4 of Video	2.66 ± 0.39	2.37 ± 0.02	3.34 ± 0.33

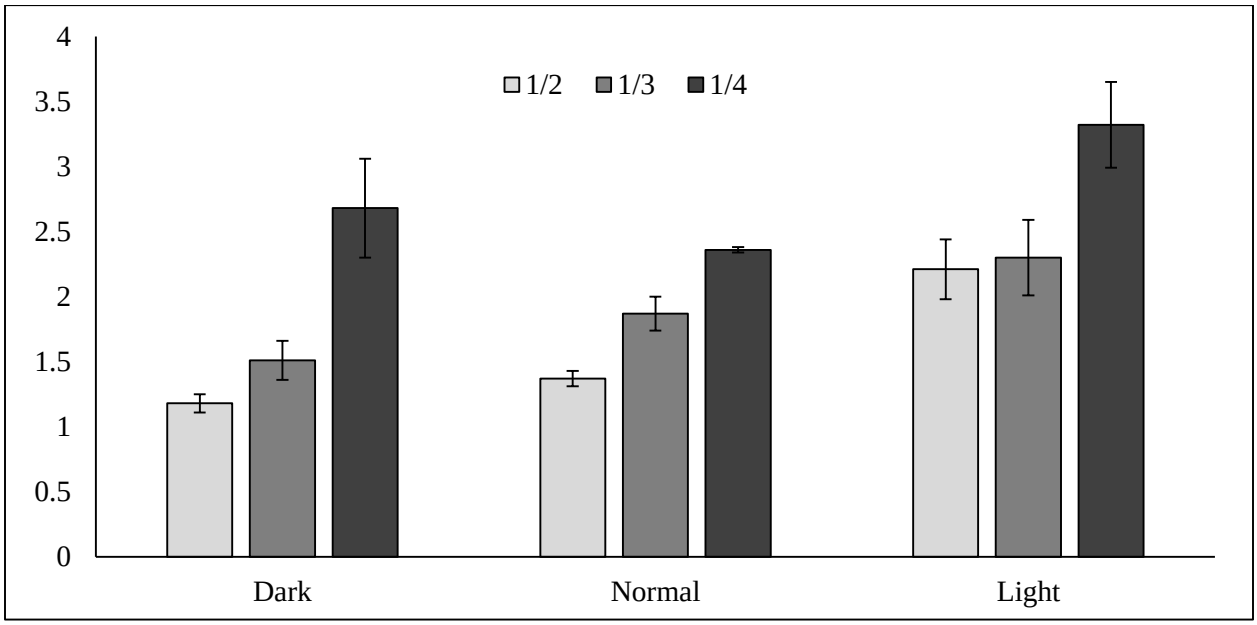


Figure 11: Compliance Comparisons for Compliance Tester Characterization.

The results of the statistical analysis can be seen below in Figures 12 and 13.

Grouping Information Using Tukey Method

	N	Mean	Grouping
smaller, light	3	3.3409	A
smaller, dark	3	2.6563	A B
smaller, normal	3	2.3706	A B C
small, light	3	2.2929	A B C D
normal, light	3	2.2507	A B C D
small, normal	3	1.8473	B C D
small, dark	3	1.4845	C D
normal, normal	3	1.3715	C D
normal, dark	3	1.1869	D

Means that do not share a letter are significantly different.

Figure 12: Grouping results of Tukey's Method. Left column presented as [size], [brightness] where Normal size is 1/2, Small is 1/3, and Smaller is 1/4.

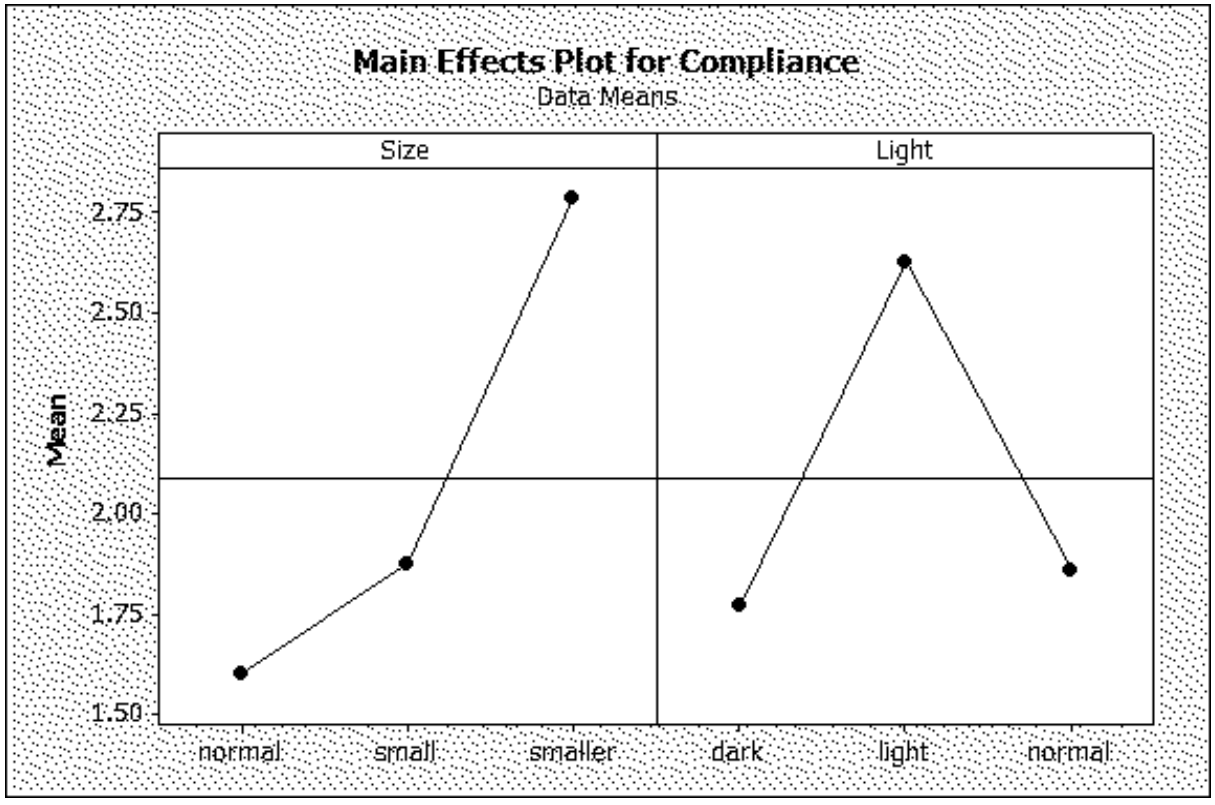


Figure 13: Main Effects Plot labeling smaller and light conditions as contributing to large variations in the measured compliance.

2.4.3 Analysis

As shown in Table IV and Figure 11, there is a trend of increasing compliance with a decrease in size and an increase in brightness, and as confirmed in Figure 13, the smaller size and lighter image both contribute to a large increase in measured compliance. However, as evinced by the Tukey's method groupings in Figure 12, the small image size has more of a significant effect than a lighter image. Although the reasons for these two trends are not known, a similarity exists between the two suggesting image size is the main factor at play: by making the image lighter, the image size effectively decreases as shown in Table IV.

Since a correct compliance value is not known for the tubing sample, it is impossible to determine which microscope configuration produces the most accurate values. However, for the sake of repeatability analysis, which is the main purpose of this piece of equipment, a particular configuration can be used so that every trial is conducted with the same settings. From the results of this testing and from qualitative usability analysis, it is recommended that compliance testing be performed with the scaffold image taking roughly 1/2 of the screen at rest, which corresponds to the largest possible image size with 4x magnification, and be performed with the lightest setting, matching the images in Table III. The large image size is recommended because the small image size seems to increase the measured compliance to unreasonable values and because the setting is easy to obtain since it is the upper limit of the focus. The light image picture is recommended because again, there is less guess-work involved in reaching this setting because there is a definite point where the "fuzz" on the edges disappears and the image of the scaffold ceases decreasing in diameter. Although these settings may not produce the true compliance, by standardizing them, the measured compliances may be compared to one another with certainty in order to evaluate scaffold variability.

Even with the diameter analysis factors controlled, there may still be variability in the data introduced by the manual pressure introduction syringe system. Since the pressure data collection and video recording cannot be controlled by the same program, and therefore cannot begin at the same time, pressure was applied and held constant prior to starting either recording to ensure the balloon was contacting the interior of the sample. This was done so that the recorded pressure stayed constant from t=0 to when pressure was increased, allowing for simple

calculation of starting pressure. However, this technique led to variations in starting pressure from 4 psi up to 10 psi since it was unknown what pressure was held until the pressure data started collecting. To detect whether these variations had an effect on the compliance values, the three compliances per each size/brightness combination were plotted versus their respective starting pressures and the linear trends were observed (Figure 14).

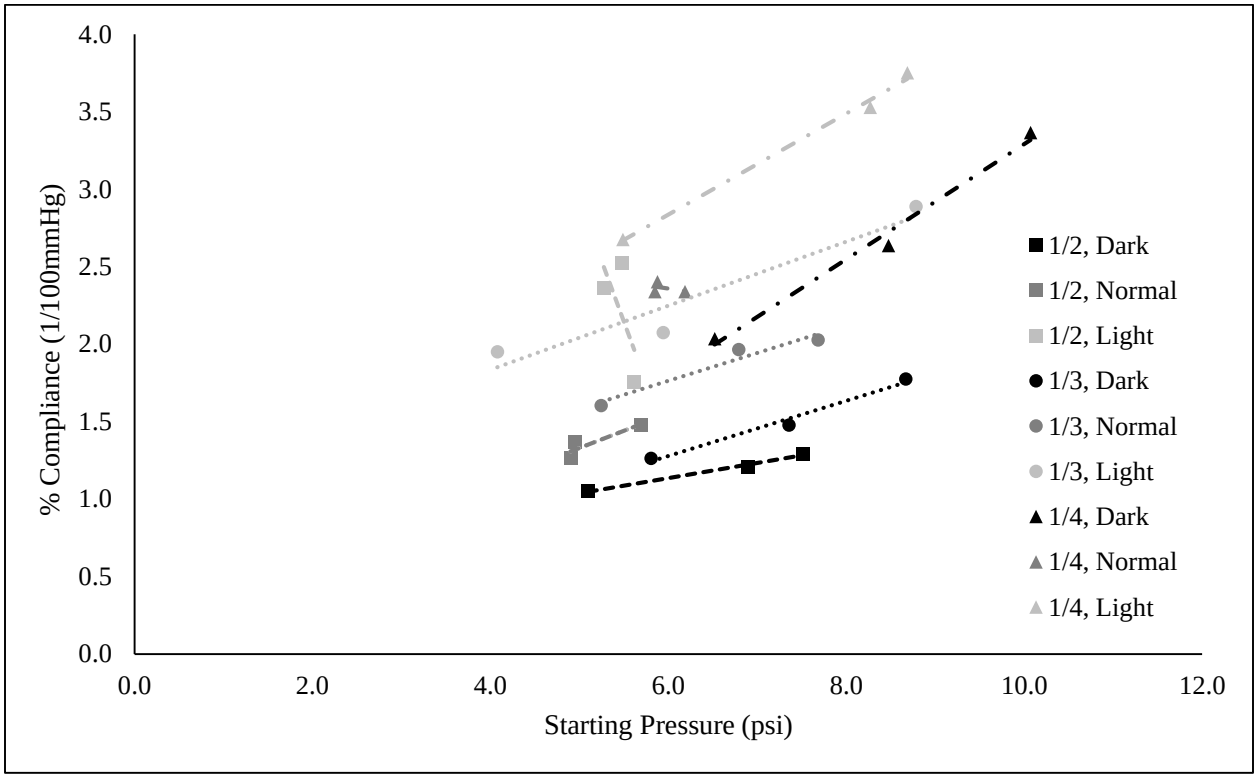


Figure 14: Investigation of the effects of starting pressure on measured compliance.

As shown in Figure 14, in all but two groups, a positive correlation was observed between starting pressure and measured compliance, with an average R^2 value for those positive correlations of 0.947. This strongly suggests that a larger starting pressure leads to a larger percent compliance. Therefore to control this, it is suggested that the protocol be changed to start the pressure recording prior to applying pressure, bring the pressure to 0.5 V or (5 psi), and then begin recording video. Then during the data analysis, the user should find the point where pressure starts increasing from 0.5 V and average the values from 10 ms prior to the increase in pressure to obtain the starting pressure. This technique should help mitigate the effects seen above in Figure 14 and further control the testing procedure.

2.5 Conclusions

Compliance is an important property to consider when evaluating tubular samples such as tissue engineered blood vessel mimics (BVMs) because it allows the sample's physical properties to be evaluated without damaging the sample as is necessary with tensile testing. For the Cal Poly Tissue Engineering Lab, compliance testing will be used to evaluate the repeatability of the BVM manufacturing process, therefore the testing itself must produce precise results. This project served to fully implement a previously-designed compliance tester with additional improvements to the physical structure, software, and protocol to ensure repeatability.

As was detailed in the previous sections, the physical improvements allowed for more repeatable placement of the sample under the microscope with the clamp and leveling of the catheter; the software improvements decreased the runtime for analysis twelve-fold while providing a more user-friendly GUI; and the testing provided protocol improvement recommendations to reduce variation due to protocol variables. The three protocol improvement suggestions are reiterated below: to test all scaffolds at maximum size (full zoom on the 4x lens) and at the brightness level where the edge blur disappears, and to apply pressure from 5 to 15 psi in every trial. As stated, these improvements are recommended to produce the most consistent results with the current equipment and set-up, however large errors may still exist and it may not produce remotely accurate results.

If accuracy is desired, the fixture and equipment should be updated in the following ways. First, instead of a manual syringe pressure introduction system, an automated dispenser should be used that can be programmed to apply pressure from 5 to 15 psi and increase that pressure at a consistent rate every trial. Second, instead of image analysis that relies on pixel counts and can be largely effected by brightness and image size, a laser micrometer should be used that can record the actual change in sample diameter. Last, a software should be developed that can control both of these devices, that the user can simply click "start" and have the pressure be automatically applied while the diameter change is recorded, and finally that can output the true percent compliance to the user. The drawback with this system is the high cost associated with the syringe pump and laser micrometer, however if true compliance is desired the costs would be justified.

3 Cryosectioning and Histological Analysis

To evaluate the BVMs for cell-sodding reproducibility, it is necessary to perform histology, which allows visual inspection of the luminal tissue lining. Histology is defined as the study of the microscopic structure of cells and tissues, which is typically accomplished by cutting very thin slices of the tissue, staining the tissue sections, and imaging the stained tissue [10]. To perform this sectioning, the tissue sample must be embedded in a solid medium, which is usually paraffin wax. However, as discussed by P. Quinn in his evaluation of cryosectioning as an alternative histological method, this method of embedding damages PLGA scaffolds due to the high temperatures required to melt the wax [10]. Thus, P. Quinn evaluated cryosectioning and concluded that it is a viable histological method that should be adopted by the Cal Poly Tissue Engineering Lab [10]. Therefore, the purpose of this work is to further P. Quinn's work and fully implement cryosectioning as the histological analysis method for BVMs produced by the Tissue Engineering Lab.

3.1 Introduction

The process of cryosectioning is very similar to the paraffin wax method in that the sample is embedded and sectioned, however instead of being embedded in wax and then sectioned at room temperature, the sample is embedded in freezing media, frozen, and sectioned at sub-freezing temperatures. Cryosectioning is often used for very delicate tissues that cannot handle the high temperatures associated with paraffin embedding (60-65°C [11]), such as brain tissue. Therefore, although frozen sections cannot be as thin as paraffin sections [12], which leads to a less-clear image of the tissue, cryosectioning is advantageous for PLGA BVMs because it does not compromise the sample by melting the scaffold (Figure 15).

Figure 15: A stained section of paraffin-embedded PLGA BVM.

The general protocol for cryosectioning involves first placing the sample into a mold containing OCT (Optimal Cutting Temperature) solution, then quick freezing the OCT/sample in liquid nitrogen to minimize ice crystal formation [12]. Once the sample is embedded, it is sectioned within a cryostat which keeps an internal temperature of roughly -20°C [12]. The sections are immediately transferred to treated slides that specialize in adhering cryosections, then the sections may be used for a variety of analysis techniques, such as immunochemistry, enzymatic detection, or simple H&E (hematoxylin and eosin) staining [12].

3.2 Methods

For the purposes of sectioning PLGA BVMs, the typical cryosectioning protocol had to be slightly modified and expanded upon in order to achieve quality sections. For reference, the entire protocol developed throughout the course of this project can be found in Appendix F. The first change that had to be made was to fix the BVMs in formalin immediately after removal from their bioreactors instead of after sectioning. This change was important because it allowed for a more flexible sectioning schedule without fear of cell migration and death after removal from the bioreactor. Once fixed, the sample could then be embedded and sectioned at any time. For embedding, following typical practice, the fixed BVMs were placed in liquid OCT solution as shown in Figure 16, then lowered into liquid nitrogen. The frozen samples were then stored in a -20°C freezer until they were sectioned.

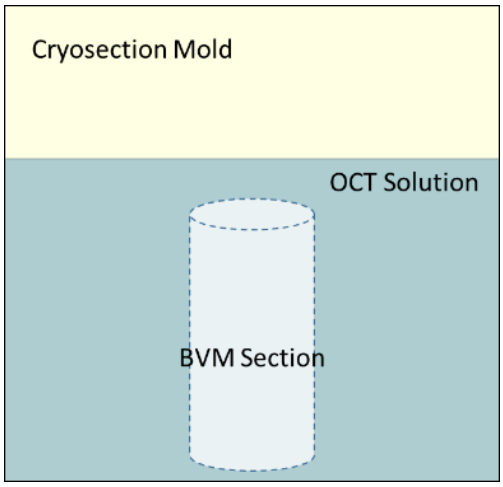


Figure 16: The orientation of a BVM section in the cryosection mold.

The next adjustment from standard protocol involved the sectioning method. As was initially discovered by P. Quinn and further confirmed by this work, the method to achieve a high-quality section was to section quickly, discard the waste produced by the fast section, then section slowly. This process was found to be much more likely to produce a high-quality section than only sectioning slowly. This phenomenon is believed to be caused by slight catching that occurs while sectioning slowly: it was noticed that the sample would "catch", evidenced by brief stopping, while automatically sectioning at a slow speed. This catching is believed to form edges on the remaining sample that only cause additional catching if another slow section is attempted. Therefore, to counter this issue—most likely caused by a dulled blade—it was necessary to perform a fast section to create a clean face for the next section.

If sections would still crumble or turn out poorly in any way, a variety of techniques were used to limited success. One method was to allow the sample to freeze for longer on the cryostat quick freeze platform (see Appendix F) to super-cool and further solidify the sample. This method was employed when the sections would consistently stick to the blade or anti-roll guard, or when the scaffold would disintegrate upon sectioning (Figure 17).

Figure 17: A section where the scaffold disintegrated during cutting.

If this was not enough to create a better section, a variety of other techniques were used including adjusting the portion of the blade used to cut, cleaning the blade and anti-roll guard with ethanol, adjusting the position of the anti-roll guard, or lifting the anti-roll guard slightly while sectioning.

Once a quality section was cut, it was transferred to a slide by contacting the section to a slide and letting it melt without allowing it to refreeze onto the blade. This process occasionally

proved tricky and led to the loss of many potentially good sections. One commonly encountered problem was one half of the scaffold did not adhere to the slide, so the surface tension of the thin film of OCT solution lifted the unattached half and folded it onto the attached half. To counter this, a rolling transfer technique was used that quickly rolled the slide so the entire section would contact the slide. This proved to significantly decrease the occurrence of section folding.

Once 12-15 quality sections were transferred onto slides (3-4 slides), the slides were stained with hematoxylin and eosin (H&E) to visually display the luminal cell lining by marking the cell nuclei (hematoxylin) and cytoplasmic proteins (eosin). This was done using a modified H&E protocol that adds drying steps to ensure section adhesion, developed by P. Quinn in his evaluation of cryosectioning (Appendix G). Once sections were stained, they were imaged with a white-light microscope.

One downside of simply performing H&E staining is the inability to distinguish smooth muscle cells (SMCs) from endothelial cells (ECs) on dual-sodded scaffolds. To visualize these cells separately, it is necessary to perform immunochemistry to tag the SMCs and ECs with different fluorescent antibodies. Since these immunochemistry protocols require substantial time and resources to perform, a “quick fix” was proposed after it was noticed that the fluorescent cell-tracker markers put on the cells prior to sodding remained fluorescent for over a week. Thus, it was proposed that imaging the cryosections for the cell tracker markers prior to staining could provide a glimpse into how the cells stacked on top of each other within the lumen. Indeed it did, as discussed in Section 3.3, which prompted a slight change to the protocol that necessitated sectioning within a week after the BVMs were removed from their bioreactors and necessitated performing a majority of the protocol in low lighting. Both of these changes were made to preserve the fluorescent marker which degrades with exposure to light. The final protocol can be found in Appendix F.

3.3 Results

The sectioning protocol implemented by this work was able to obtain good quality sections at 15μm, which is better than the previous standard set by P. Quinn at 20 μm [10]. However, some sections, although intact, showed signs of damage from the sectioning process (Figure 18).

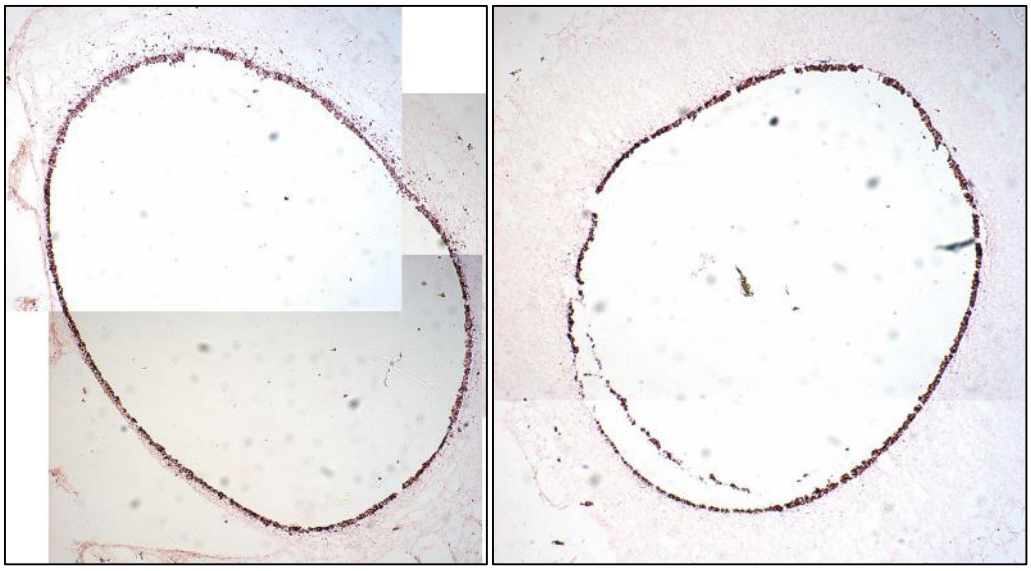


Figure 18: Histological sections of PLGA BVMs obtained through cyrosectioning. Left, a representative high-quality section; Right, a section with damage from sectioning procedure.

These cutting artifacts were observed on a number of sections and should be an area of additional investigation as to why some sections did not have damage while others did. One possibility is a thicker scaffold could lead to a more rigid structure that prevents damage as it was observed that thicker scaffolds tended to produce better sections.

The cell-tracker imaging of scaffolds proved crucial to the lab progress by giving insight into how the pressure-sodding technique for both ECs and SMCs led to poor linings. The cell-tracker images showed how ECs were only found in clumps within the scaffold, past the layer of previously sodded SMCs (Figure 19), which prompted the switch from pressure-sodding to gravity-sodding.

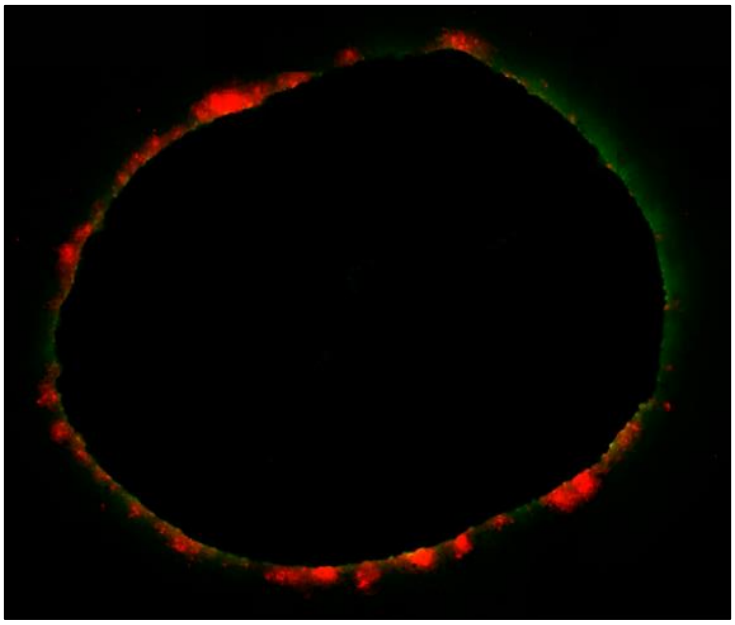


Figure 19: A cross-sectional cell tracker image showing the clumps of ECs (red) behind the SMC (green) layer due to pressure-sodding.

The new gravity-sodding procedure allowed the ECs to lay on top of, instead of squeezed through, the SMC layer which produced more physiologically-similar linings of ECs exterior to a layer of SMCs (Figure 20).

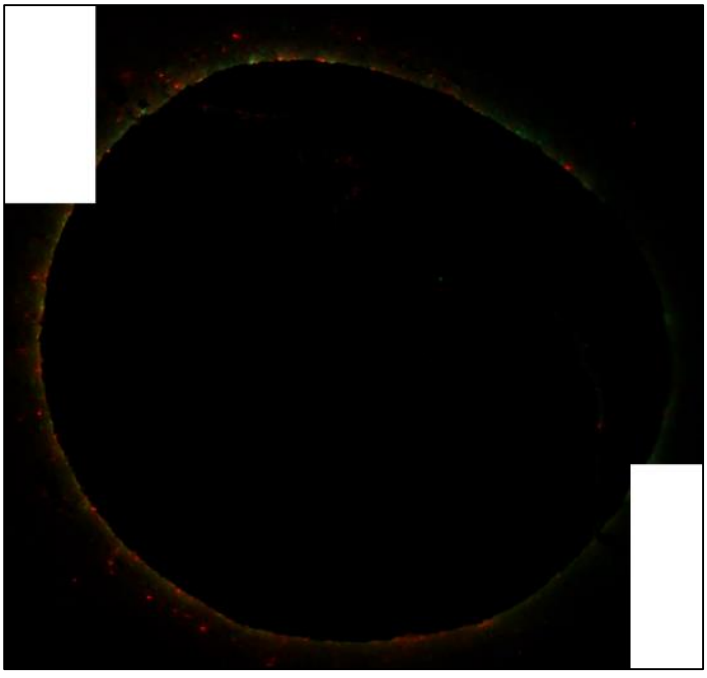


Figure 20: A cross-sectional cell tracker image showing the more evenly distributed ECs (red) on top of the SMCs (green) due to gravity-sodding.

3.4 Conclusions

From the development of the cryosectioning protocol and observing the resulting stained sections, many potential improvements and directions for future work became apparent. With the prevalence of samples that were difficult to section or that produced damaged sections, the first and primary direction for future work is investigating the various factors that could be involved in section quality. From the limited observations obtained while implementing the protocol, it seems that thicker scaffolds, samples that have been allowed to freeze longer, and the shorter the fixation period lead to higher-quality sections. However, these claims are simple observations and must be substantiated by data collection of fixation times, freezing times, external temperatures, and scaffold thicknesses among other potential factors. One improvement that was made at the end of this project was sharpening the blade which hadn't been sharpened in four years. This will hopefully reduce many of the problems experienced during the course of this project, however there is the potential that it will not.

While imaging the sections for the cell-tracker markers, cross-fluorescence was observed where ECs slightly fluoresced like SMCs and vice versa. This fluorescent "bleeding" indicates that the cell-tracker markers may not be adequate to display the different cell layers with any certainty. Therefore, additional staining procedures to selectively mark SMCs and ECs should be investigated if the lab hopes to definitively prove that the BVM manufacturing process repeatedly produces physiologically-similar luminal blood vessel linings.

4 Conclusions and Future Work

This work served to increase the blood vessel mimic (BVM) evaluation capacity of the Cal Poly Tissue Engineering Lab for the purpose of verifying manufacturing repeatability. To evaluate the physical property of compliance, a previously-constructed compliance test fixture and software were further developed and implemented to reduce measurement variability and increase throughput. To evaluate cell-sodding results, a previously-developed cryosectioning protocol was further improved and implemented to repeatedly produce high-quality BVM sections and tissue stains. However, with the implementation of BVM analysis techniques, a number of improvements or areas of future investigation became apparent. These have been previously discussed in the appropriate sections, but are summarized below for convenience.

4.1 Compliance

With a functioning compliance tester, the lab now has the capability to evaluate BVM scaffold strength properties pre- and post-sodding in order to further characterize scaffold manufacturing parameters, and to understand how conditioning and tissue-sodding affects the scaffold properties. This will give the lab additional data when characterizing the manufacturing process, and could eventually be used as a monitoring test to ensure scaffold manufacturing consistency with the establishment of acceptable compliance ranges. However, there are still improvements that could be made to the tester.

First, the current protocol should be adjusted to apply pressure after starting pressure recording, bring the pressure to 5 psi (0.5V), and then begin the video recording. Then to find the starting pressure during analysis, the user should find the point in the pressure file where the pressure starts to increase above 0.5V and average the pressures for 10 ms prior to this point. This improvement will serve to limit the observed effects of different starting pressures on the calculated compliance values, and with this change, the current protocol would optimize the fixture's repeatability.

The final recommended improvements deal with a complete revision of the fixture hardware and software in order to accurately measure compliance data for comparison to actual blood vessels, since the current setup does not allow for the calculation of true compliance values. In order to do so would first require a more reliable diameter measurement instead of using basic image analysis that is dependent on pixel counts, image size, and brightness. The recommended upgrade would be a laser micrometer to measure the actual diameter of the sample so that a true percent change in diameter could be calculated. With the diameter calculation controlled, the next improvement would be a programmable syringe pump to control the pressure application. This improvement would serve to avoid the variability associated with starting pressures, pressure application rate, and final pressures introduced through human error. The final improvement would be to develop software to control both the laser micrometer and the syringe pump so that pressure application initiates at the same time as diameter recording.

4.2 Cryosectioning

With a functional cryosectioning protocol, the lab now has the ability to obtain histological sections of the BVMs, which allows for future development of staining protocols to image the deposited tissue. The development of these imaging procedures will allow the lab to view the various cell types and ensure the BVMs in fact mimic the native blood vessel tissue. However, although a protocol has been established, there are further improvements to be made.

The most important direction for future work with cryosectioning is to fully investigate the factors that contribute to section quality. From the limited observations made in the course of this project, a few potential candidates include scaffold thickness, OCT-embedded sample temperature, time in fixative, tissue coverage, and ambient air temperature while sectioning. By characterizing these factors' effects on section quality, a protocol can be developed with a higher rate of producing quality sections.

The second direction for future work involves investigating various procedures for selectively staining SMCs and ECs with higher resolution than the cell tracker marker. By developing a working protocol, the two cell types would be distinguished on a section with certainty and the burden of embedding and cryosectioning in low lighting would be alleviated.

5 Acknowledgements

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Appendix

A References

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B Component Data Sheets

RUGGED, GENERAL PURPOSE TRANSDUCER

COMMON SPECIFICATIONS

mV Output Wiring

Wiring	Cable	M12 and mini DIN	Twist-Lock
Excitation (+)	Red	Pin 1	Pin A
Output (+)	White	Pin 3	Pin C
Output (-)	Green	Pin 4	Pin D
Excitation (-)	Black	Pin 2	Pin B
Spare			Pin E
Vent			Pin F

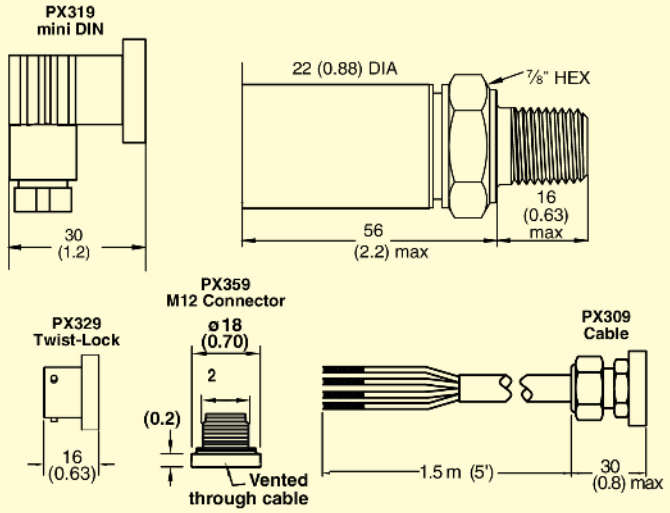
5 Vdc Output Wiring

Wiring	Cable	M12 and mini DIN	Twist-Lock
Excitation (+)	Red	Pin 1	Pin A
Excitation (-)	Black	Pin 2	Pin B
Output (+)	White	Pin 3	Pin C
N/C[1]		Pin 4	Pin D
Spare			Pin E
Vent			Pin F

mA Output Wiring

Wiring	Cable	M12 and mini DIN	Twist-Lock
Supply (+)	Red	Pin 1	Pin A
Supply (-)	Black	Pin 2	Pin B
N/C[1]		Pin 3	Pin C
N/C[1]		Pin 4	Pin D
Spare			Pin E
Vent			Pin F

[1] *N/C: Do not connect any wires to this pin.*



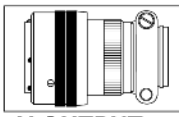
SPECIFICATIONS

OMEGA's PX309 Series models below 100 psi use a high-accuracy silicon sensor protected by an oil-filled stainless steel diaphragm. Units 100 psi and above use silicon strain gages molecularly bonded to the stainless steel diaphragm

Long-Term Stability (1 Year): ±0.25% typical
Typical Life: 10 million cycles typical
Operating Temperature: -40 to 85°C (-40 to 185°F)
Proof Pressure:
All psia and ≤50 psig Ranges: 3x capacity or 20 psi, whichever is greater
100 psig Ranges: 2x capacity
Burst Pressure: 500% of capacity or 25 psi, whichever is greater
Response Time: <1 ms
Shock: 50 g, 11 ms half-sine
Vibration: ±20 g
Protection Class: IP 65
Wetted Parts:
316 SS for all psia and 1 to 50 psig ranges; 17-4 PH stainless steel for ranges 100 to 10,000 psig
Pressure Port: ¼-18 MNPT
Electrical Connections:
PX309: 1.5 m (5') 2-, 3-, or 4-conductor cable (mA, 5V, mV outputs, respectively)
PX319: mini DIN connector with mating connector included
PX329: Twist-lock connector, vented mating connector sold separately (PT06V-10-6S)
PX359: M12, 4-pin connector
Weight:
PX309: 154 g (5.4 oz)
PX319, PX329, PX359: 100 g (3.5 oz)

100 mV OUTPUT

Excitation:
0 to 50 psig and All psia Ranges: 10 Vdc (ratiometric), (5 to 12 Vdc limits)
100 to 10,000 psig Ranges: 5 Vdc (ratiometric), (3 to 10 Vdc limits)
Output: 0 to 100 mV, except 2 psi = 40 mV and 1 psi = 20 mV
Accuracy: ±0.25% FS BSL at 25°C; includes linearity, hysteresis and repeatability
Zero Offset: ±2% FSO; ±4% for 1 and 2 psi ranges
Span Setting: ±2% FSO; ±4% for 1 and 2 psi ranges
Compensated Temperature: 0 to 50°C (32 to 122°F)
Thermal Zero and Span Effects (Over Compensated Range):
15 to 10,000 psi Ranges: ±2% FSO
5 psi Range: ±3% FSO
2 psi Range: ±4% FSO
1 psi Range: ±5% FSO



PX329 Mating Connector PT06V-10-6S

5V OUTPUT

Excitation: 9 to 30 Vdc (reverse polarity and overvoltage protected)
Output: 0 to 5 Vdc or 4 to 20 mA
Static Accuracy 5 to 10,000 psi: ±0.25% FS BSL at 25°C; includes linearity, hysteresis and repeatability
Zero Offset: ±2% FSO; ±4% for 1 and 2 psi ranges
Span Setting: ±2% FSO; ±4% for 1 and 2 psi ranges
Compensated Temperature:
>5 psi Range: -20 to 85°C (-4 to 185°F)
≤5 psi Range: 0 to 50°C (32 to 122°F)
Total Error Band: ±2% FSO; includes linearity, hysteresis, repeatability, thermal hysteresis and thermal errors (except 2 psi = ±3% and 1 psi = ±4.5%)

Order a snubber to protect your pressure transducer!

PS-4G, shown actual size.

Snubbers protect sensors from fluid hammers/spikes.

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- **High Stability, Low Drift**
- **0.25% Static Accuracy**

5V OUTPUT SPECIFICATIONS

Excitation: 9 to 30 Vdc (reverse polarity and overvoltage protected)
Output: 0 to 5 Vdc or 4 to 20 mA
Static Accuracy 5 to 10,000 psi: ±0.25% FS BSL at 25°C; includes linearity, hysteresis and repeatability
Zero Offset: ±2% FSO; ±4% for 1 and 2 psi ranges
Span Setting: ±2% FSO; ±4% for 1 and 2 psi ranges
Compensated Temperature:
>5 psi Range: -20 to 85°C (-4 to 185°F)
≤5 psi Range: 0 to 50°C (32 to 122°F)
Total Error Band: ±2% FSO; includes linearity, hysteresis, repeatability, thermal hysteresis and thermal errors (except 2 psi = ±3% and 1 psi = ±4.5%)

To Order

RANGE bar	RANGE psi	1.5 m CABLE CONNECTION	MINI DIN CONNECTION	TWIST-LOCK CONNECTION
ABSOLUTE PRESSURE				
0 to 0.34	0 to 5	PX309-005A5V	PX319-005A5V	PX329-005A5V
0 to 1	0 to 15	PX309-015A5V	PX319-015A5V	PX329-015A5V
0 to 2.1	0 to 30	PX309-030A5V	PX319-030A5V	PX329-030A5V
0 to 3.4	0 to 50	PX309-050A5V	PX319-050A5V	PX329-050A5V
0 to 6.9	0 to 100	PX309-100A5V	PX319-100A5V	PX329-100A5V
0 to 14	0 to 200	PX309-200A5V	PX319-200A5V	PX329-200A5V
0 to 21	0 to 300	PX309-300A5V	PX319-300A5V	PX329-300A5V
GAGE PRESSURE				
0 to 0.07	0 to 1	PX309-001G5V	PX319-001G5V	PX329-001G5V
0 to 0.14	0 to 2	PX309-002G5V	PX319-002G5V	PX329-002G5V
0 to 0.34	0 to 5	PX309-005G5V	PX319-005G5V	PX329-005G5V
0 to 1	0 to 15	PX309-015G5V	PX319-015G5V	PX329-015G5V
0 to 2.1	0 to 30	PX309-030G5V	PX319-030G5V	PX329-030G5V
0 to 3.4	0 to 50	PX309-050G5V	PX319-050G5V	PX329-050G5V
0 to 6.9	0 to 100	PX309-100G5V	PX319-100G5V	PX329-100G5V
0 to 10	0 to 150	PX309-150G5V	PX319-150G5V	PX329-150G5V
0 to 14	0 to 200	PX309-200G5V	PX319-200G5V	PX329-200G5V
0 to 21	0 to 300	PX309-300G5V	PX319-300G5V	PX329-300G5V
0 to 34	0 to 500	PX309-500G5V	PX319-500G5V	PX329-500G5V
0 to 69	0 to 1000	PX309-1KG5V	PX319-1KG5V	PX329-1KG5V
0 to 138	0 to 2000	PX309-2KG5V	PX319-2KG5V	PX329-2KG5V
0 to 207	0 to 3000	PX309-3KG5V	PX319-3KG5V	PX329-3KG5V
0 to 345	0 to 5000	PX309-5KG5V	PX319-5KG5V	PX329-5KG5V
0 to 517	0 to 7500	PX309-7.5KG5V	PX319-7.5KG5V	PX329-7.5KG5V
0 to 690	0 to 10,000	PX309-10KG5V	PX319-10KG5V	PX329-10KG5V

Comes complete with 5-point NIST-traceable calibration.

Notes: *1. Units 100 psig and above may be subjected to vacuum on the pressure port without damage.*
2. For alternative performance specifications to suit your application, contact Engineering.

Ordering Examples: PX309-100G5V, *100 psi gage pressure transducer with 0 to 5 Vdc output and 1.5 m cable termination.*

PX319-015A5V, *15 psi absolute pressure transducer with 0 to 5 Vdc output and mini DIN termination.*

PX329-3KG5V, *3000 psi gage pressure transducer with 0 to 5 Vdc output and twist-lock termination. Mating connector sold separately; order* ***PT06V-10-6S.*** *Consult Sales for OEM pricing.*

ACCESSORIES

MODEL NO.	DESCRIPTION
CAL-3	Recalibration: 5-point NIST traceable
PT06V-10-6S	Mating connector for PX329
CA-329-4PC24-005	4-conductor mating twist-lock connector with 1.5 m (5') cable for PX329
CX5302	Extra mini DIN connector for PX319

Specifications

GENERAL

Isolation: 500V from PC

External Excitation Output Voltage: 12 Vdc regulated, max total current output 67 mA

Power Requirements: Powered direct from USB port, max 500 mA, or from external 7.5 to 12 Vdc

Environmental: 0 to 50°C (0 to 122°F) 95% RH (non-condensing)

Operating Temperature: 0 to 50°C (32 to 122°F), 0 to 95% RH non-condensing

Storage Temperature: -40 to 85°C (-40 to 185°F)

Weight: 0.23 kg (0.5 lb)

Dimensions: 107 W x 128 L x 39 mm H (4.2 x 5.1 x 1.5")

Input Voltage Range: Software programmable on a per-channel basis differential/single-ended

-10	to 10V	-500 to 500 mV
-5	to 5V	-250 to 250 mV
-2.5	to 2.5V	-125 to 125 mV
-2	to 2V	-75 to 75 mV
-1	to 1V	-30 to 30 mV

TC Input Range

Type J: -18 to 1200°C (0 to 2192°F)

Type K: -129 to 1372°C (-200 to 2502°F)

Type T: -101 to 400°C (-150 to 752°F)

Type E: -184 to 1000°C (-300 to 1832°F)

Type R: 204 to 1768°C (400 to 3214°F)

Type S: 204 to 1768°C (400 to 3214°F)

Type B: 538 to 1820°C (1000 to 3308°F)

Type N: -129 to 1300°C (-200 to 2372°F)

TC Input

Thermocouple Accuracy: Typical, in very slow mode, 24 bit resolution

J = ±1.1°C
K = ±1.2°C
T = ±1.1°C
E = ±1.0°C
R = ±2.5°C
S = ±2.6°C
B = ±3.3°C
N = ±1.5°C

Cold Junction Compensation Accuracy: ±1.0°C

Analog Input Accuracy:

Differential Input: Typical, in very slow mode, 0.015% of reading +0.004% of range +10uV (exclusive of noise)

Single-End Input: Typical, in very slow mode, 0.05% of reading +0.01% of range +50uV (exclusive of noise)

USB Device Type: USB 2.0 (full-speed)

Device Compatibility: USB 1.1, USB 2.0

Power Supply: From USB or 9 Vdc universal adaptor (included)

DIN Rail Mounted for Rack Application: Optional

Open Thermocouple Detect: Automatically enable when a channel is configured for a thermocouple sensor

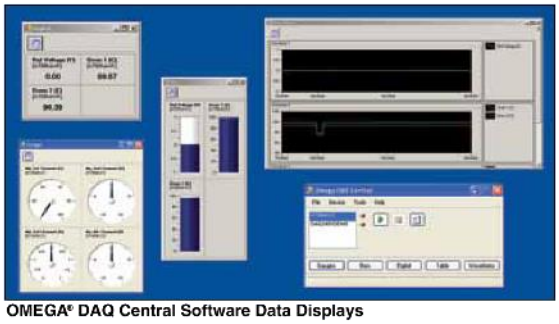
The OM-DAQ-USB-2401 comes complete with hardware for both DIN rail and wall mounting, both shown here.

OMEGA® DAQ Central Software Device Controls

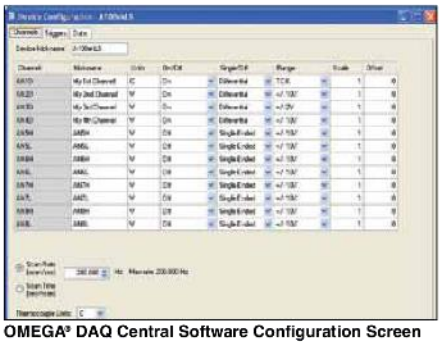
OMEGA® DAQ Central Software

Each OM-DAQ-USB-2401 comes complete with an impressive FREE, easy-to-use software suite that allows charting, data logging, file storage and virtual instrument viewing of data in real time. Users can use the provided .NET driver for C#, Visual Basic and Visual C++ to design and develop their own application software.

A demo version of DAQ central can be downloaded from omega.com/software

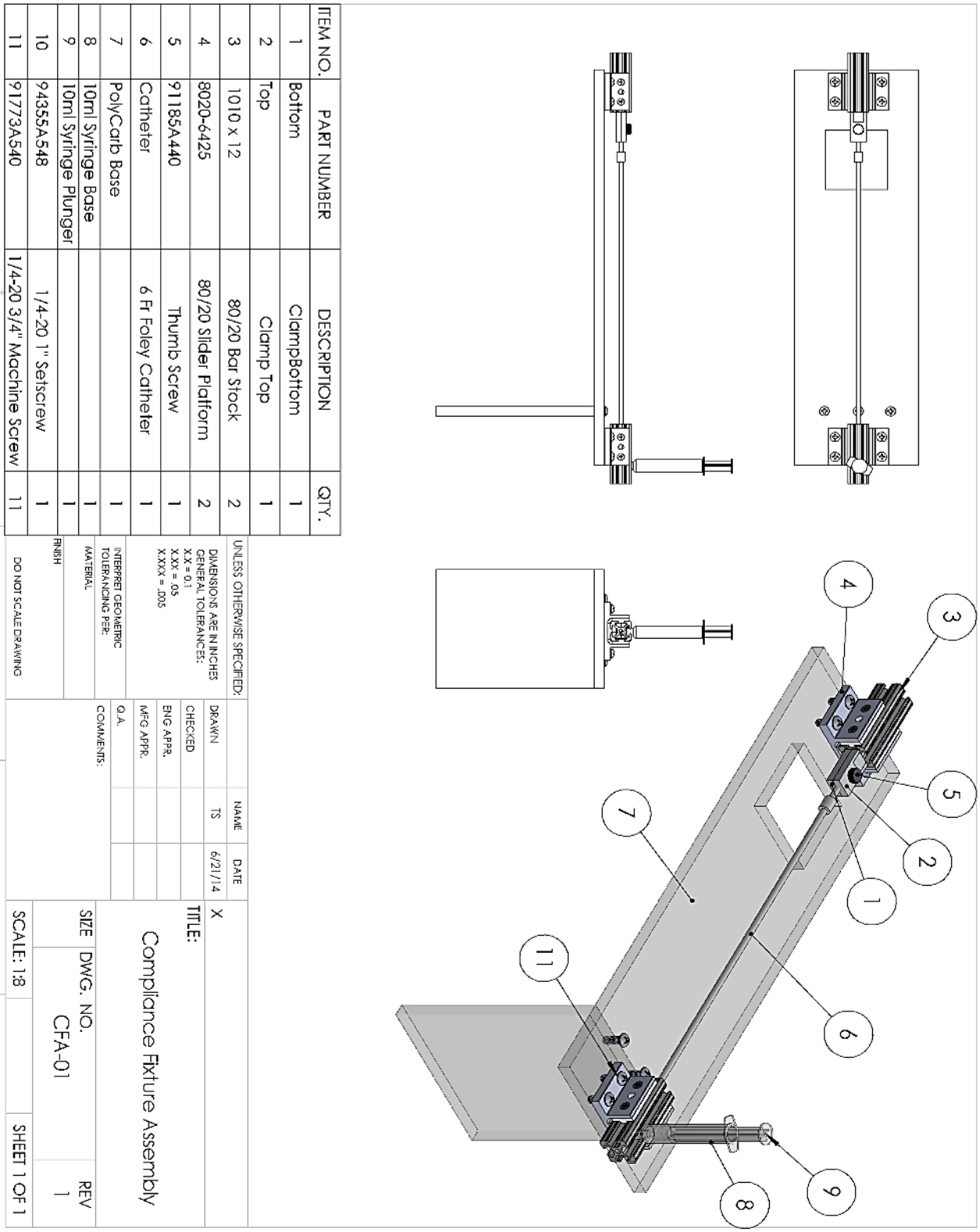


OMEGA® DAQ Central Software Data Displays



OMEGA® DAQ Central Software Configuration Screen

C Detailed Drawings



ITEM NO.	PART NUMBER	DESCRIPTION	QTY.
1	Bottom	ClampBottom	1
2	Top	Clamp Top	1
3	1010 x 12	80/20 Bar Stock	2
4	8020-6425	80/20 Slider Platform	2
5	91185A440	Thumb Screw	1
6	Catheter	6 Fr Foley Catheter	1
7	PolyCarb Base		1
8	10ml Syringe Base		1
9	10ml Syringe Plunger		1
10	94355A548	1/4-20 1" Setscrew	1
11	91773A540	1/4-20 3/4" Machine Screw	11

UNLESS OTHERWISE SPECIFIED:
DIMENSIONS ARE IN INCHES
GENERAL TOLERANCES:
X.X = 0.1
X.XX = .05
X.XXX = .005
INTERPRET GEOMETRIC TOLERANCING PER:
MATERIAL
FINISH
DO NOT SCALE DRAWING

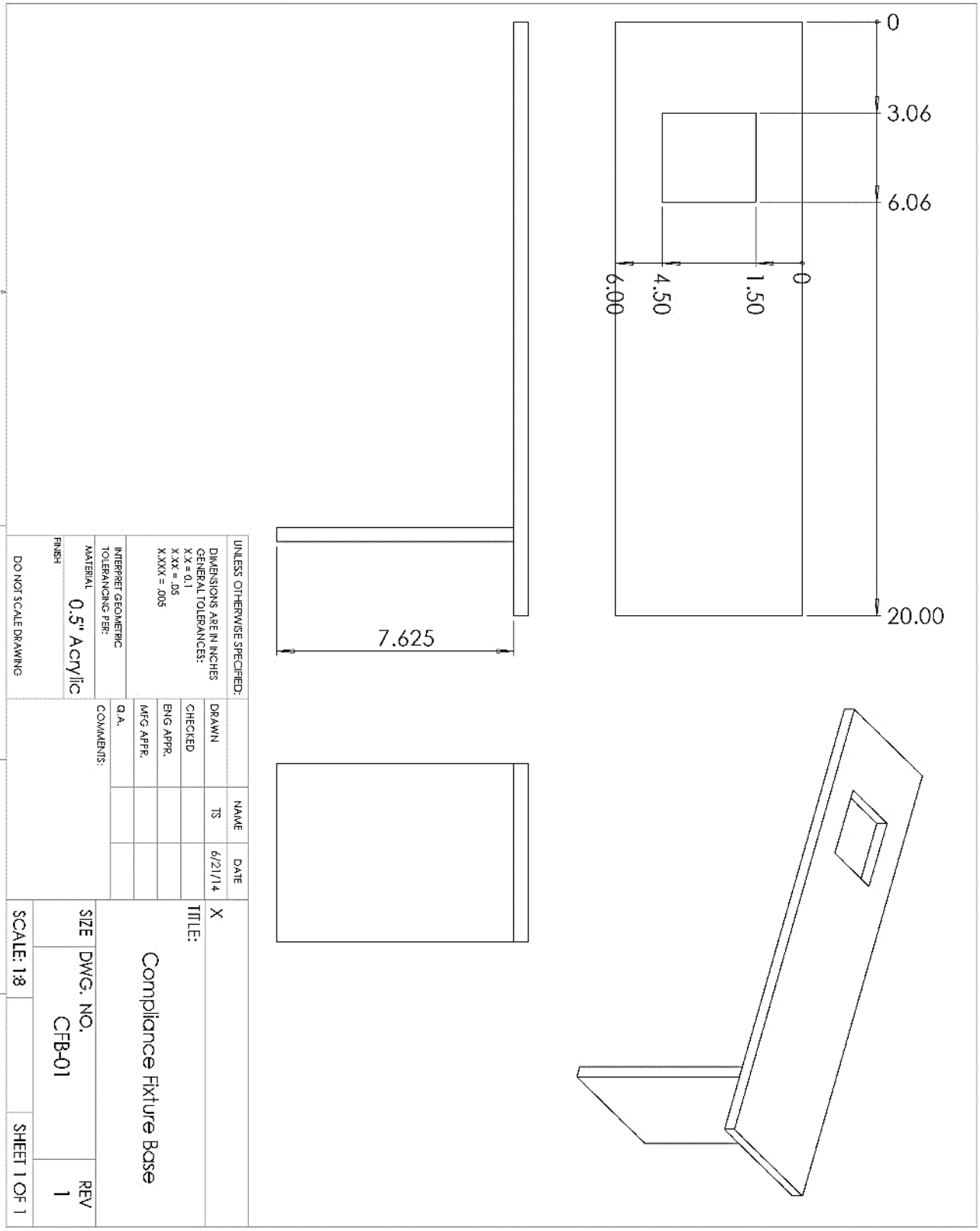
	NAME	DATE
DRAWN	TS	6/21/14
CHECKED		
ENG APPR.		
MFG APPR.		
Q.A.		
COMMENTS:		

X

TITLE:
Compliance Fixture Assembly

SIZE DWG. NO. REV
CFA-01 1

SCALE: 1:8 SHEET 1 OF 1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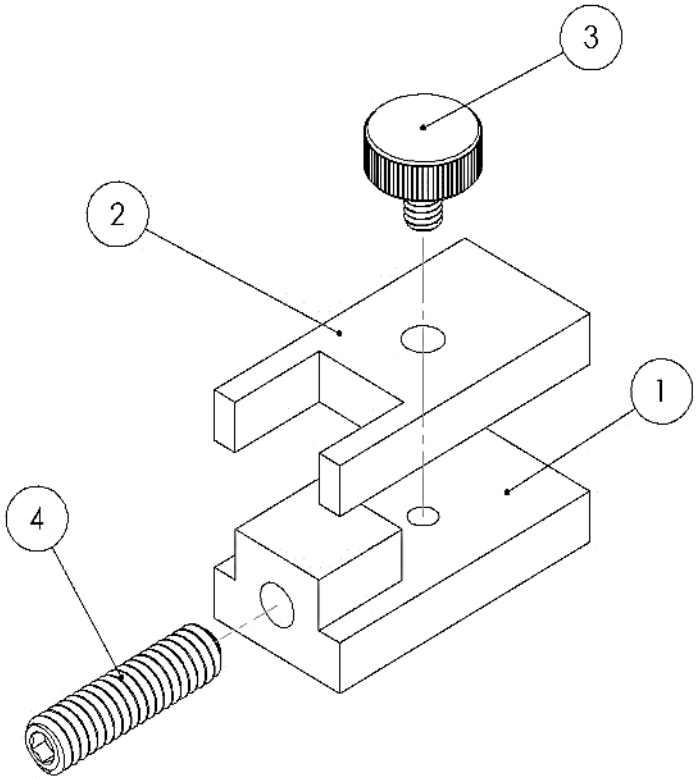
3.06
6.06
20.00
0
1.50
4.50
6.00
7.625
UNLESS OTHERWISE SPECIFIED:
DIMENSIONS ARE IN INCHES
GENERAL TOLERANCES:
X.X = 0.1
X.XX = .05
X.XXX = .005
INTERPRET GEOMETRIC TOLERANCING PER:
MATERIAL 0.5" Acrylic
FINISH
DO NOT SCALE DRAWING
NAME
DATE
DRAWN TS 6/21/14
CHECKED
ENG APPR.
MFG APPR.
Q.A.
COMMENTS:
TITLE:
Compliance Fixture Base
SIZE X
DWG. NO. CFB-01
REV 1
SCALE: 1:8
SHEET 1 OF 1



ITEM NO.	PART NUMBER	DESCRIPTION	QTY.
1	Bottom	ClampBottom	1
2	Top	Clamp Top	1
3	91185A440	Thumb Screw	1
4	94355A548	1/4-20 1" Setscrew	1

UNLESS OTHERWISE SPECIFIED:		NAME	DATE
DIMENSIONS ARE IN INCHES GENERAL TOLERANCES: X.X = 0.1 X.XX = .05 X.XXX = .005	DRAWN	TS	6/21/14
	CHECKED		
	ENG APPR.		
	MFG APPR.		
INTERPRET GEOMETRIC TOLERANCING PER:	Q.A.		
MATERIAL	COMMENTS:		
FINISH			
DO NOT SCALE DRAWING			

X

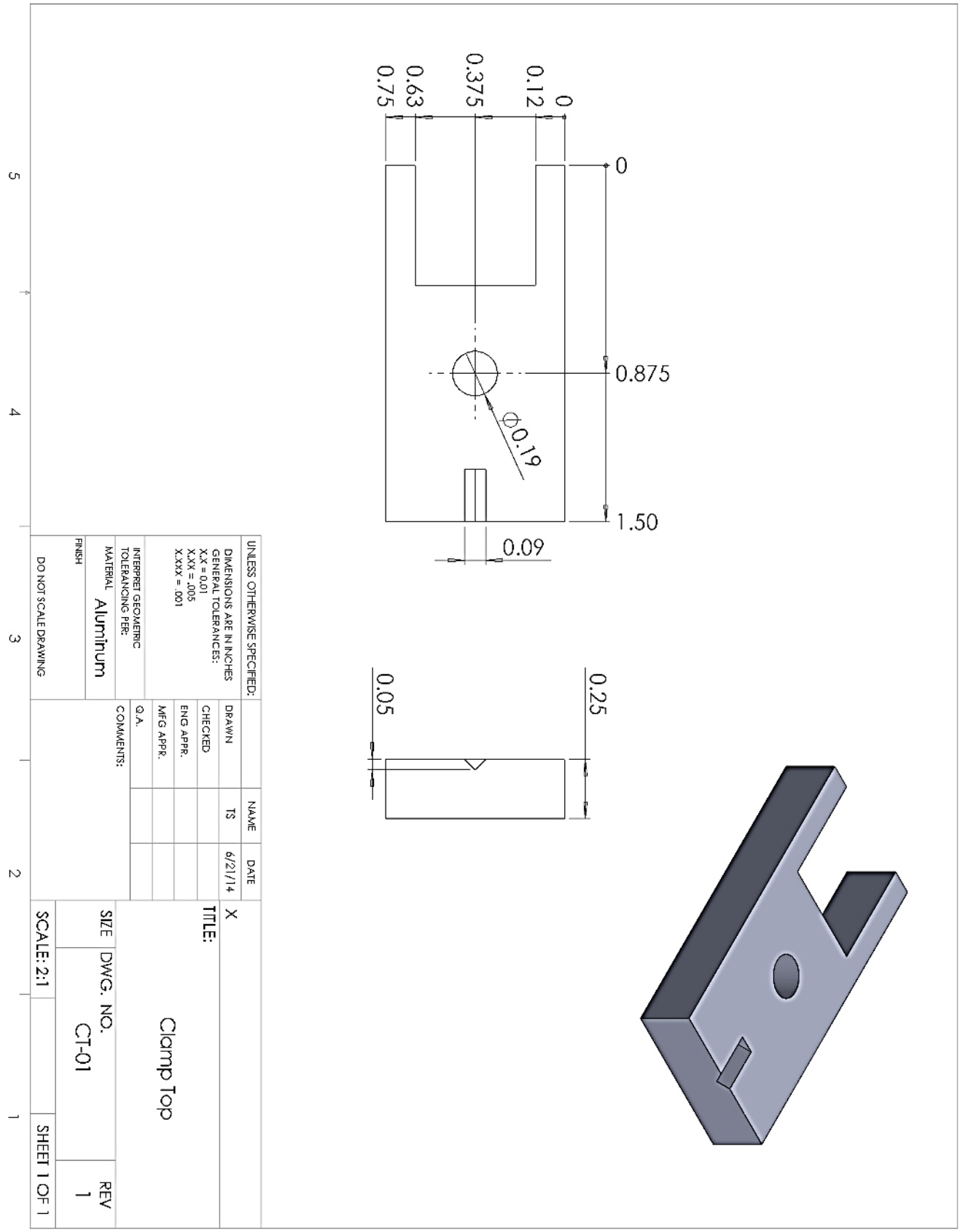
TITLE:

Catheter Clamp Assembly

SIZE	DWG. NO.	REV
	CCA-01	1

SCALE: 1:1		SHEET 1 OF 1

5 4 3 2 1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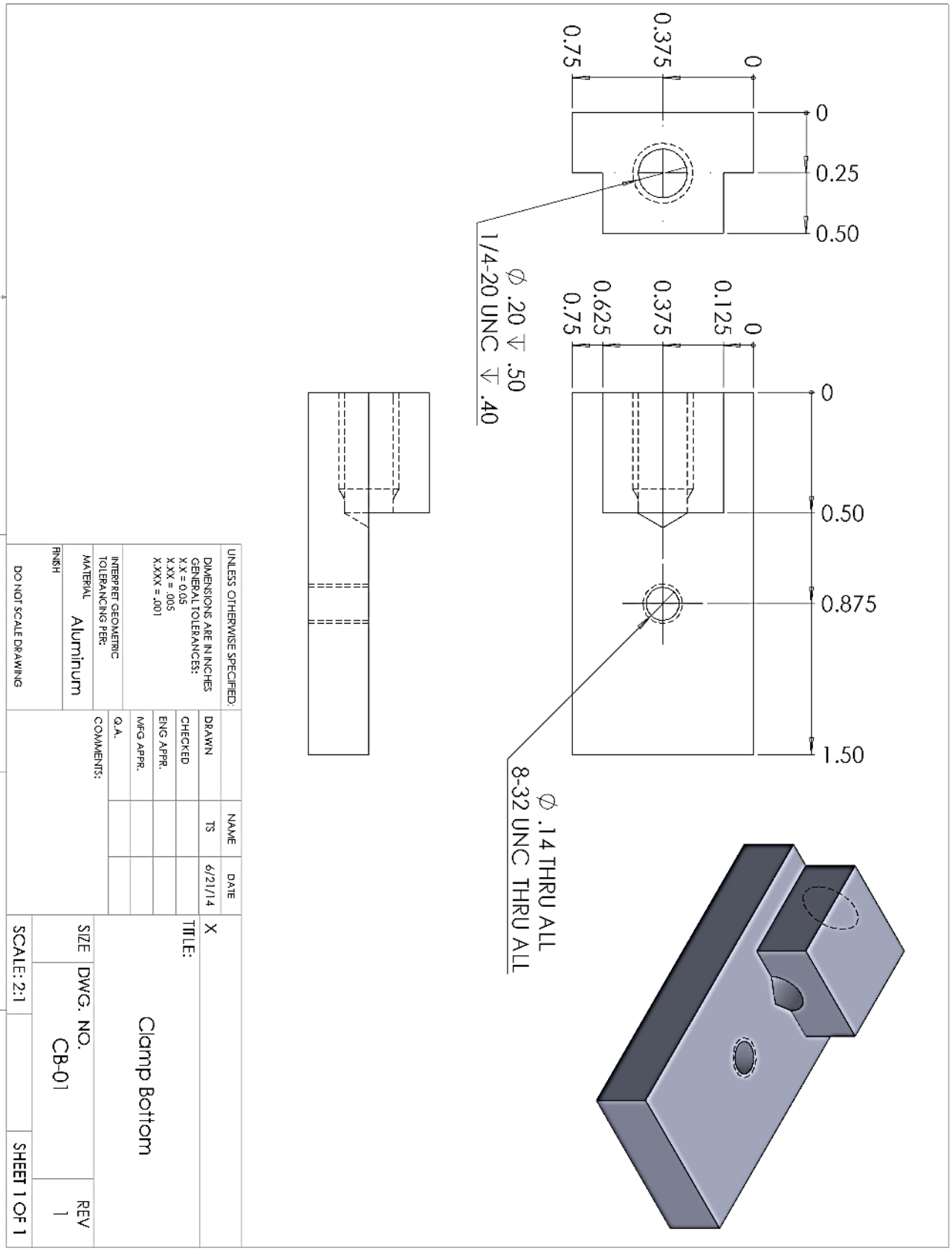
0.12
0.375
0.63
0.75
0
0.875
1.50
Φ0.19
0.09
0.25
0.05
UNLESS OTHERWISE SPECIFIED:
DIMENSIONS ARE IN INCHES
GENERAL TOLERANCES:
X.X = 0.01
X.XX = .005
X.XXX = .001
INTERPRET GEOMETRIC TOLERANCING PER:
MATERIAL Aluminum
FINISH
DO NOT SCALE DRAWING
NAME
DATE
DRAWN TS 6/21/14
CHECKED
ENG APPR.
MFG APPR.
Q.A.
COMMENTS:
X
TITLE:
Clamp Top
SIZE
DWG. NO. CT-01
REV 1
SCALE: 2:1
SHEET 1 OF 1


0
0.375
0.75
0
0.25
0.50
Ø .20 ⩛ .50
1/4-20 UNC ⩛ .40
0
0.125
0.375
0.625
0.75
0
0.50
0.875
1.50
Ø .14 THRU ALL
8-32 UNC THRU ALL
UNLESS OTHERWISE SPECIFIED:
DIMENSIONS ARE IN INCHES
GENERAL TOLERANCES:
X.X = 0.05
X.XX = .005
X.XXX = .001
INTERPRET GEOMETRIC TOLERANCING PER:
MATERIAL
Aluminum
FINISH
DO NOT SCALE DRAWING
DRAWN
TS
6/21/14
CHECKED
ENG APPR.
MFG APPR.
Q.A.
COMMENTS:
X
TITLE:
Clamp Bottom
SIZE
DWG. NO.
CB-01
REV
1
SCALE: 2:1
SHEET 1 OF 1

D Compliance Code

```
function ComplianceGUI

    %COMPLIANCEGUI View live video, record video, and analyze the video

    % Make GUI figure
    f = figure('Visible', 'off', 'Position', [200, 200, 1122, 628],...
        'CloseRequestFcn', @my_closereq, 'Color',[1 1 1],...
       'IntegerHandle', 'off', 'MenuBar', 'none', 'ToolBar', 'none',...
       'Name','Compliance Tester', 'NumberTitle', 'off');

    % Make "start camera" button
    hStart = uicontrol('Style', 'pushbutton', 'String', 'Start Camera',...
                'Position', [50, 510, 200, 50], 'Callback', @startCamera);
    % Make "capture video" button
    hCapture = uicontrol('Style', 'pushbutton', 'String', 'Capture Video',...
                'Position', [50, 410, 200, 50], 'Callback', @captureVideo);
    % Make "replay video" button
    hReplay = uicontrol('Style', 'pushbutton', 'String', 'Replay Video',...
                'Position', [50, 310, 200, 50], 'Callback', @replayVideo);
    % Make "analyze video" button
    hAnalyze = uicontrol('Style', 'pushbutton', 'String', 'Analyze Video',...
                'Position', [50, 210, 200, 50], 'Callback', @analyzeVideo);
    % Make "% Change in Diameter" title
    hText = uicontrol('Style', 'edit', 'String', '% Change in Diameter',...
                'Position', [50, 110, 200, 50], 'BackgroundColor', [0.8, 0.8,
0.8]);
    % Make output box for % change in diameter result
    hResult = uicontrol('Style', 'edit', 'Position', [50, 60, 200, 50]);
    % Make the viewer window
    hViewer = axes('Units','pixels','Position',[300,19,788,591], 'Box',...
        'on', 'ytick',[],'xtick',[]);

    % Create video object
    video = videoinput('winvideo', 2);

    % Initialize the video to get a snapshot from the camera every 0.05s
    set(video,'TimerPeriod', 0.05, 'TimerFcn', @getVideo);

    % Configure the video trigger to turn on and off manually (records video)
    triggerconfig(video,'manual');
    video.FramesPerTrigger = Inf; % Capture frames until we manually stop it

    % Turn capture video button off
    set(hCapture,'Enable','off');

    movegui(f, 'center');
    set(f, 'Visible', 'on');

    % CALLBACK FUNCTION
    function getVideo(src, eventdata)
    % Function to retrieve and display video data within the viewing window
        snap = getsnapshot(video); % Get picture using GETSNAPSHOT
```

```
        imshow(snap) % and put it into axes using imshow
    end

    % CALLBACK FUNCTION
    function startCamera(src, eventdata)
    % Function to Start/Stop the Camera

        set(hCapture,'Enable','off');
        set(hReplay,'Enable','off');
        set(hStart, 'Enable','off');
        set(hAnalyze, 'Enable','off');

        if strcmp(get(hStart,'String'),'Start Camera')
              % Camera is off. Change button string and start camera.
              set(hStart,'String','Stop Camera')
              start(video)
              set(hCapture,'Enable','on'); %allow user to capture video
        else
              % Camera is on. Stop camera and change button string.
              set(hStart,'String','Start Camera')
              stop(video)
              set(hCapture,'Enable','off'); %turn off capture button
              blankimage = zeros(591,788);
              imshow(blankimage)
        end

        set(hReplay,'Enable','on');
        set(hStart, 'Enable','on');
        set(hAnalyze, 'Enable','on');
    end

    % CALLBACK FUNCTION
    function captureVideo(src, eventdata)
        % Function to start/stop video acquisition

        % Turn off unneccessary warning
        warning('off', 'imaq:getdata:infFramesPerTrigger');

        set(hCapture,'Enable','off');
        set(hReplay,'Enable','off');
        set(hStart, 'Enable','off');
        set(hAnalyze, 'Enable','off');

        if strcmp(get(hCapture,'String'),'Capture Video')
       % Camera is not acquiring. Change button string and start acquisition.
              set(hCapture,'String','End Capture');
              trigger(video);
        else
              % Camera is acquiring. Stop acquisition, save video data,
              % and change button string.
              stop(video);
              [Filename, Pathname] =
uiputfile('C:\Users\student\Desktop\*.avi');

              if Pathname ~= 0
```

```
                cd(Pathname); % change directory to save the file to

                % Code to write captured video to avi
                movWriter = VideoWriter(Filename);
                open(movWriter);
                videoData = getdata(video);
                writeVideo(movWriter, videoData);
                close(movWriter);
            end

            start(video); % Restart the camera
            set(hCapture,'String','Capture Video');
            set(hReplay,'Enable','on');
            set(hStart, 'Enable','on');
            set(hAnalyze, 'Enable','on');
    end

    set(hCapture,'Enable','on');
end

% CALLBACK FUNCTION
function replayVideo(src, eventdata)
    % Function to replay the video
    set(hCapture,'Enable','off');
    set(hReplay,'Enable','off');
    set(hStart, 'Enable','off');
    set(hAnalyze, 'Enable','off');

    % check if video feed is already on
    if strcmp(get(hStart,'String'),'Stop Camera')
        stop(video); % if it is then stop the camera
    end

    % get filename of video to play
    [Filename, Pathname] = uigetfile('C:\Users\student\Desktop\*.avi');

    if Pathname ~= 0
        cd(Pathname);

        % create video object to read from
        movObj = VideoReader(Filename);
        %lastFrame = read(movObj, inf);
        nFrames = movObj.NumberOfFrames;

        for k = 1 : nFrames
            %isolate frame 1-by-1
            singleFrame = read(movObj, k);

            % show the image in the GUI
            imshow(singleFrame)
        end
    end

    % check if the camera was on
```

```
    if strcmp(get(hStart,'String'),'Stop Camera')
        start(video); % if it was then restart the camera
        set(hCapture,'Enable','on');
    else
        blankimage = zeros(591,788);
        imshow(blankimage)
    end

    set(hReplay,'Enable','on');
    set(hStart, 'Enable','on');
    set(hAnalyze, 'Enable','on');
end

% CALLBACK FUNCTION
function analyzeVideo(src, eventdata)
    % Function to analyze diameter change in tubular samples
    % to determine percent compliance

    set(hCapture,'Enable','off');
    set(hReplay,'Enable','off');
    set(hStart, 'Enable','off');
    set(hAnalyze, 'Enable','off');

    % check if video feed is already on
    if strcmp(get(hStart,'String'),'Stop Camera')
        stop(video); % if it is then stop the camera
    end

    % Open user-specified video
    [Filename, Pathname] = uigetfile('C:\Users\student\Desktop\*.avi');

    if Pathname ~= 0
        cd(Pathname);
        diamChange = VideoReader(Filename); %create videoReader object

        % get video data
        %lastFrame = read(diamChange, inf);
        nframes = diamChange.NumberOfFrames;
        frameWidth = get(diamChange, 'Width');
        frameHeight = get(diamChange, 'Height');

        initialDiam = 0.0;
        maxDiam = 0.0;

        for k = 1 : nframes
            %isolate frame 1-by-1 and convert it to grayscale
            singleFrame = read(diamChange, k);
            grayFrame = rgb2gray(singleFrame);

            % find threshold level and convert to binary image
            level = graythresh(grayFrame);
            bw = im2bw(grayFrame, level);

            % show the image
```

```
                imshow(bw)

                % calculate diameter of scaffold in frame
                currentDiam = (frameWidth*frameHeight -
bwarea(bw))/frameWidth;

                % if it is the first frame
                if k == 1
                    initialDiam = currentDiam; %save that diameter as the
initial
                end

                % save new max diameter
                if currentDiam > maxDiam
                    maxDiam = currentDiam;
                end
            end

            % Calculate percent change in diamter and print out to gui
            percentChange = (maxDiam - initialDiam)/initialDiam * 100;
            set(hResult, 'String', num2str(percentChange));
        end

        % check if the camera was on
        if strcmp(get(hStart,'String'),'Stop Camera')
            start(video); % if it was then restart the camera
            set(hCapture,'Enable','on');
        else
            blankimage = zeros(591,788);
            imshow(blankimage)
        end

        set(hReplay,'Enable','on');
        set(hStart, 'Enable','on');
        set(hAnalyze, 'Enable','on');
    end

    % UIWAIT makes ComplianceGUI wait for user response (see UIRESUME)
    uiwait(f);
end

function my_closereq(src, eventdata)
% User-defined close request function
% to display a question dialog box
   selection = questdlg('Close Compliance Tester GUI?',...
      'Close Request',...
      'Yes','No','Yes');
   switch selection,
      case 'Yes',
         delete(src)
         delete(imaqfind)
      case 'No'
      return
   end
end
```

E Compliance Testing Protocol

1. Identify the Olympus CKX41 microscope and compliance tester fixture. Make sure everything is plugged in to the power strip. (Figure 1).

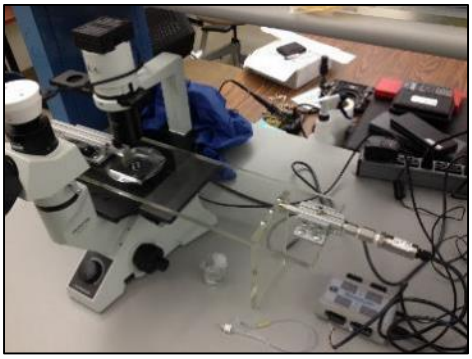


Figure 1: The Olympus CKX41 Microscope and compliance testing fixture.

2. Turn the microscope on. Verify that the microscope and Omega DAQ Board are plugged into a laptop with Omega DAQ Central software and LumeneraLite drivers installed via the USB ports.
3. Verify that the transducer wires are connected as shown in Figure 2.

Figure 2: DAQ wires. The proper DAQ connections.

4. Push the condenser all of the way to the left (1) and move the aperture knob (2) to the left so the light is brightest (Figure 3). Turn the microscope objective to 4x.

Figure 3: Light condenser. Image of shutter and filter in proper placement.

5. Connect the occlusion catheter to the male luer fitting as seen in Figure 4.

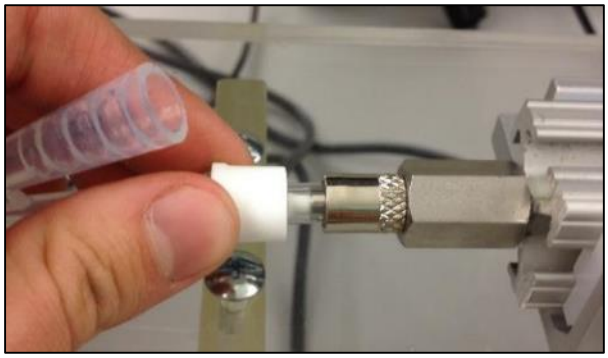


Figure 4: Catheter connection. Attach the catheter to the male luer.

6. Fill the 10mL syringe with DI water and screw it on the female luer fitting, as seen in Figure 5.

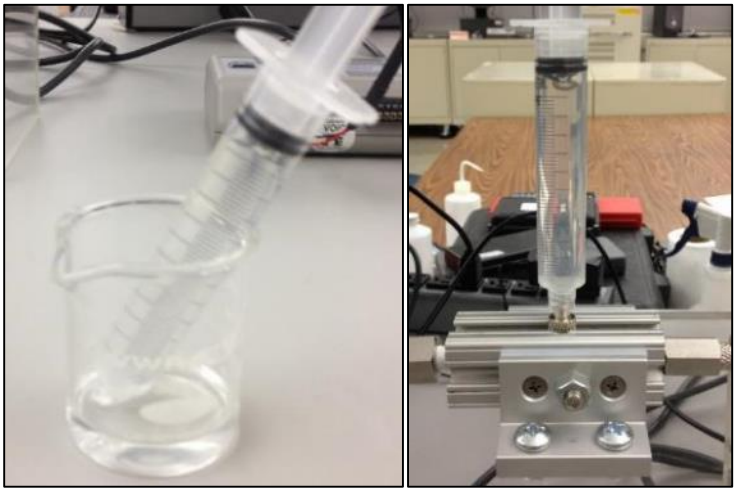


Figure 5: Syringe connection. Image of fluid-filled syringe screwed into the female luer fitting.

7. Cut <u>one</u> ~2 cm-length tubing sample. The sample length should be just long enough to cover the inflation zone; not any longer (see below).
8. Load the first tubing sample onto the catheter, making sure it is covering the inflation zone (Figure 6).

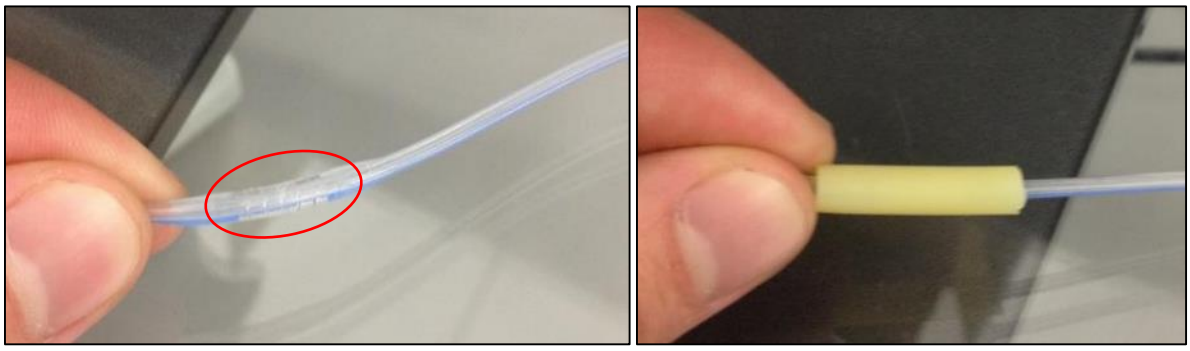


Figure 6: Image of balloon region (left) and the tubing sample over the balloon region (right).

9. Place the tip of the catheter into the clamp just until the hole in the catheter is covered. Tighten the thumb screw all the way (Figure 7).

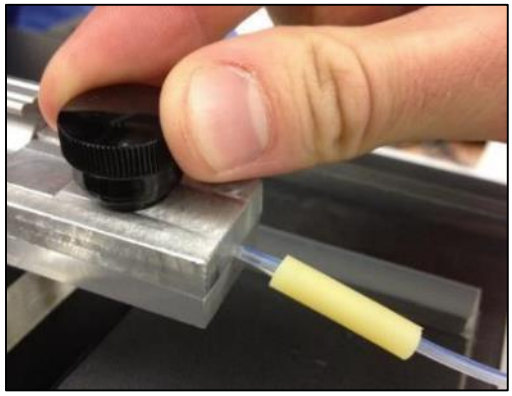


Figure 7: Placement of the catheter in the clamp.

10. Slide the clamp back so the black line is aligned with the edge of the platform (Figure 8).

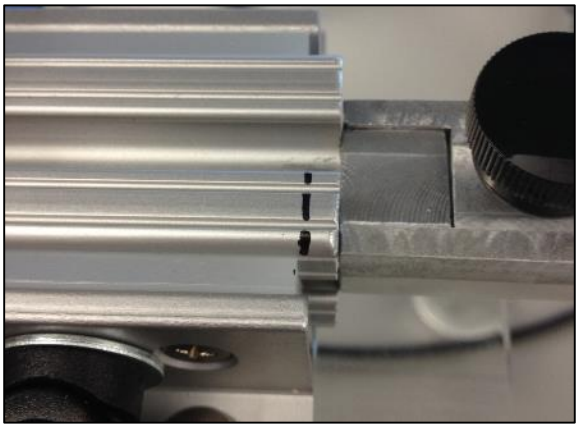


Figure 8: Location of slider when pulled back.

11. Position the fixture so that the tubing sample is roughly centered over the objective lens.
12. Open up the "DAQ Central" software package by clicking on the icon seen in Figure 9.

Figure 9: An image of the icon used to open up the DAQ program.

13. From the DAQ main window (Figure 10), click "Devices" and then "Detect Devices". The device "N0903212012" should appear in the window: this is the pressure transducer. NOTE: If the device does not appear, ensure that the green power LED is lit up on the DAQ unit, that the USB cable is plugged in, and that the wires are correctly hooked up per step 3.

Figure 10: DAQ Program. The pressure transducer can be seen highlighted in blue.

14. Click "Devices" and then "Configuration". From the menu seen in Figure 11, make sure that the AN1D channel is set to "ON", "Differential", and "+/- 5V". Also make sure the Scan Rate is set to 100 Hz.

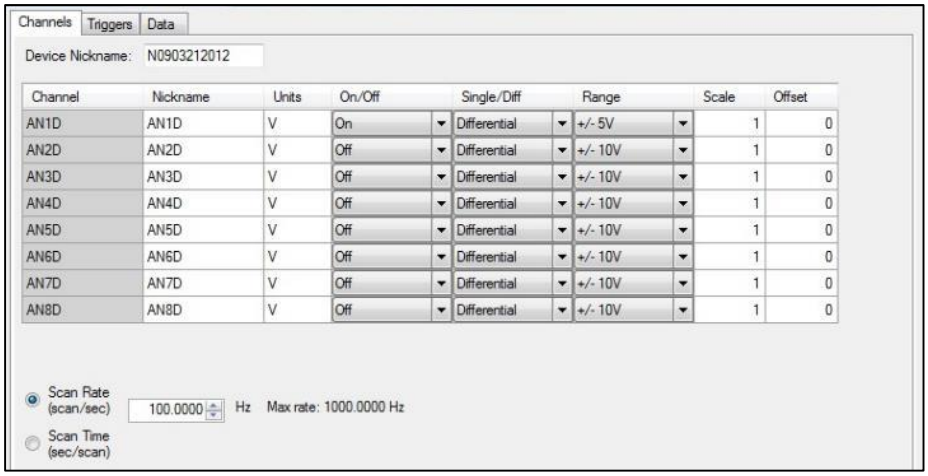


Figure 11: DAQ Configuration. The correct configuration for the pressure transducer.

15. From the same window, click the "Data" tab. As seen in Figure 12, set up the correct parameters. For "File", create a unique file name in the "Pressure Files" folder on the desktop where you can easily access the Excel file that will be created.

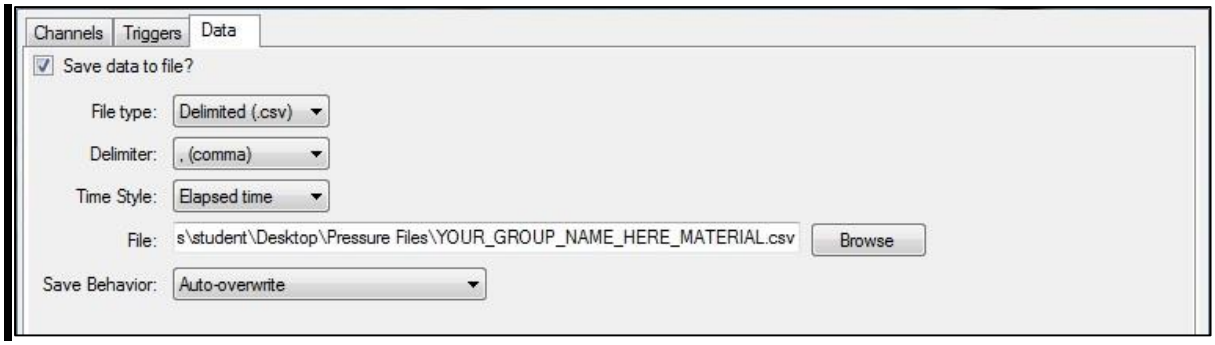


Figure 12: DAQ Data settings. An image of the correct data parameters.

16. From the main DAQ menu, hit the "play" button as seen in Figure 10. Then hit the "Digital" button. The image seen in Figure 13 should appear. This displays the voltage that the transducer is reading. "Prime" the system by applying a bit of pressure to the system and releasing (about 0.5 V). Hit the "stop" button.

Figure 13: The DAQ Voltage Display.

17. Click on the compliance shortcut on the desktop to open the compliance tester GUI.

Figure 14: The compliance tester GUI shortcut.

18. Wait for the GUI to open as shown in Figure 15.

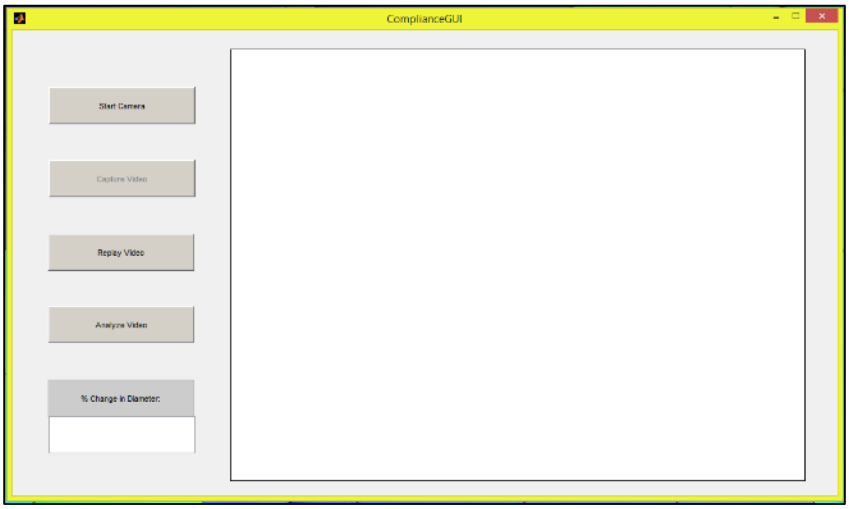


Figure 15: The compliance tester GUI

19. Turn on the camera by pressing "Start Camera".
20. Use the video feed to position the scaffold in the viewing window. Note: The video feed will lag behind your motions, so be slow, and wait for the video to "catch up" with your motions. Make the sample as horizontal in the video as possible.

21. Adjust the focus so that the scaffold image is roughly 1/2 of the video (for the smallest sample, this is the largest image possible). Adjust the lighting so that the "fuzziness" around the edges is eliminated, which occurs when the diameter ceases to decrease with increasing brightness (Figure 16).

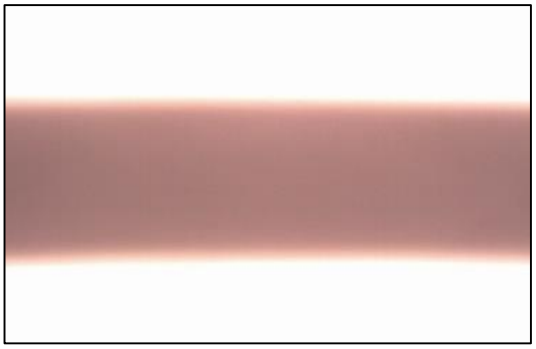


Figure 16: An example of an acceptable image of the tubing.

22. While watching the video, apply pressure to the syringe and verify the sample expands evenly within the video window. If not, adjust accordingly.
23. Now you are ready to capture the video of the pressure application. First, before starting the DAQ acquisition, apply a little bit of pressure on the syringe and hold it steady. This is to ensure the balloon is contacting the sample wall.
24. In quick succession, hit the "play" button for the DAQ pressure sensor (Step 5), then hit the "Capture Video" button on the compliance GUI.
25. Carefully apply pressure to avoid spiking the pressure reading and moving the fixture. Bring the voltage to around 1.5V and hold it there for 3 seconds.
26. Relieve the pressure then hit the "End Capture" button on the compliance GUI and the "stop" button on the DAQ software. Save the video with a unique name for ease of finding.
27. Verify the excel file was created for the pressure readings. Verify that the video was captured properly by clicking "Replay Video" and selecting the video you just saved.
28. Analyze the recorded video by clicking "Analyze Video" and selecting the appropriate video file. The video will look similar to Figure 17. Once complete, record the percent diameter change output.

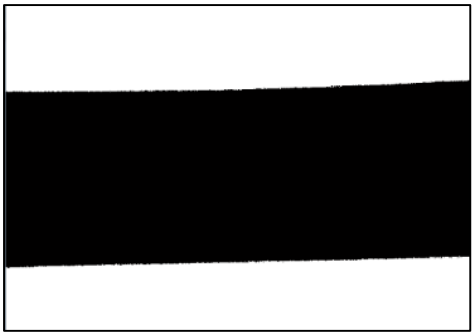


Figure 17: This is a visualization the image analysis used to calculate diameter change. The program counts the number of black pixels (representing the scaffold) and divides by the resolution in the horizontal direction to obtain a diameter value.

29. Open the excel file you saved from DAQ central.
30. Record the maximum voltage value that was achieved.
31. For the baseline pressure, average the voltages from 0 to 0.1 seconds. Compute the change in voltage.
32. Convert this change in voltage to a change in PSI by multiplying by 10 (a number provided by OMEGA). Convert this change in PSI to a change in mmHg (51.7149326 mmHg/psi).
33. Type the value from the "% Change in Diameter" output window on the compliance GUI into excel. Divide this value by the change in pressure, and multiply by 100. Units are % compliance/100 mmHg.

F Cryosectioning Protocol

Embedding

1. Retrieve covered conicals (Figure 1).

Figure 1: Covered conicals containing BVMs in fixative.

2. Obtain 1 cryosection mold for each sample and cut one lip off of each with scissors (Figure 2).

Figure 2: Cryosection mold with one lip cut off

3. Obtain liquid nitrogen in dewar.
4. Gather molds (1), sample conicals (2), 12” forceps (3), 4” forceps (4), the dewar (5), a marker (6), OCT solution (7), aluminum foil (8), and a piece of paper towel (9) on a workspace (Figure 3).

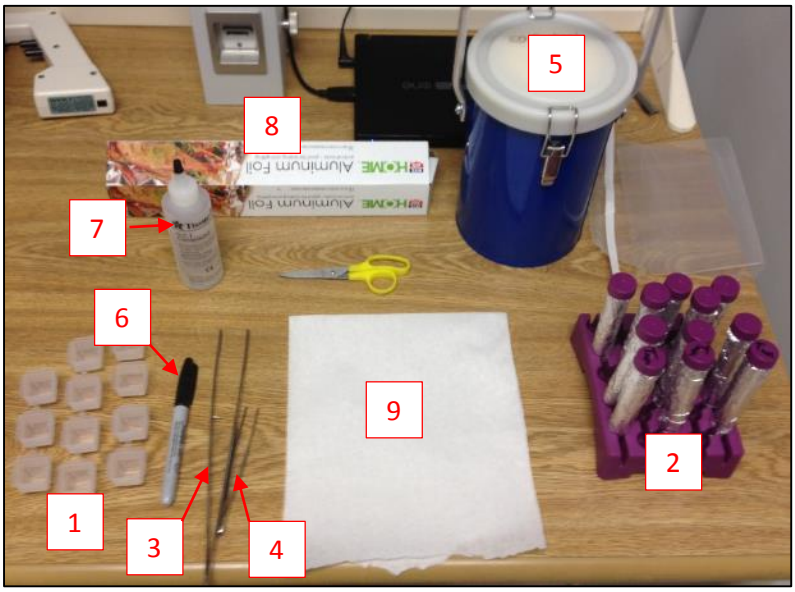


Figure 3: Embedding equipment set up on benchtop. Molds (1), sample conicals (2), 12” forceps (3), 4” forceps (4), the dewar (5), a marker (6), OCT solution (7), aluminum foil (8), and a piece of paper towel (9).

Perform the following steps in low lighting to preserve the fluorescent cell tracker marker.

5. Remove aluminum foil from conical.
6. Record the date code, letter, and initials on one mold with marker (Figure 4).

Figure 4: BVM information copied onto the cryosection mold

7. Pour OCT solution into mold until there is about a ¼” layer (Figure 5).

Figure 5: Mold with a small layer of OCT solution.

8. Remove the sample from the conical with 12” forceps and place the narrow side down into the OCT solution (Figure 6).

Figure 6: Removing the BVM from its conical and placing into the OCT solution.

9. With 4” forceps, hold the BVM vertical and pour additional OCT solution into the BVM lumen until OCT covers the entire height of the BVM.
10. With the 4” forceps, gently grab the outside of the BVM and move through the OCT solution to ensure no air bubbles remain inside the lumen.
11. Place the BVM vertically with the narrow portion pressed against the center of the mold bottom.
12. With 12” forceps, grasp the mold on the cut edge, and slowly lower into the dewar until hissing sound is heard, meaning the mold has contacted the liquid nitrogen (Figure 7).

Figure 7: Holding the mold inside the dewar with the 12” forceps.

13. Hold the mold steady until a majority of the OCT solution is frozen (about 30-45s).
14. Remove the mold from the dewar and wrap it in aluminum foil, and place into the -40°C freezer for storage (Figure 8).

Figure 8: Frozen mold wrapped in aluminum foil and placed into the freezer.

15. Repeat steps 5 through 14 for all remaining samples.
16. Replace all equipment and dispense fixative solution into the mixed organic preservative waste bin.
17. Dry the conicals in the hood and dispose of them into the biohazard bin.

Cryosectioning

1. Keep frozen molds covered and cold during transport to the cryostat.
2. Sign into the cryostat log book.
3. Prepare the cryostat for sectioning
   a. Remove the cryostat lid and place aside (Figure 9).

Figure 9: Removing the cryostat lid

   b. Identify the various parts of the cryostat for future use as described in Figure 10.

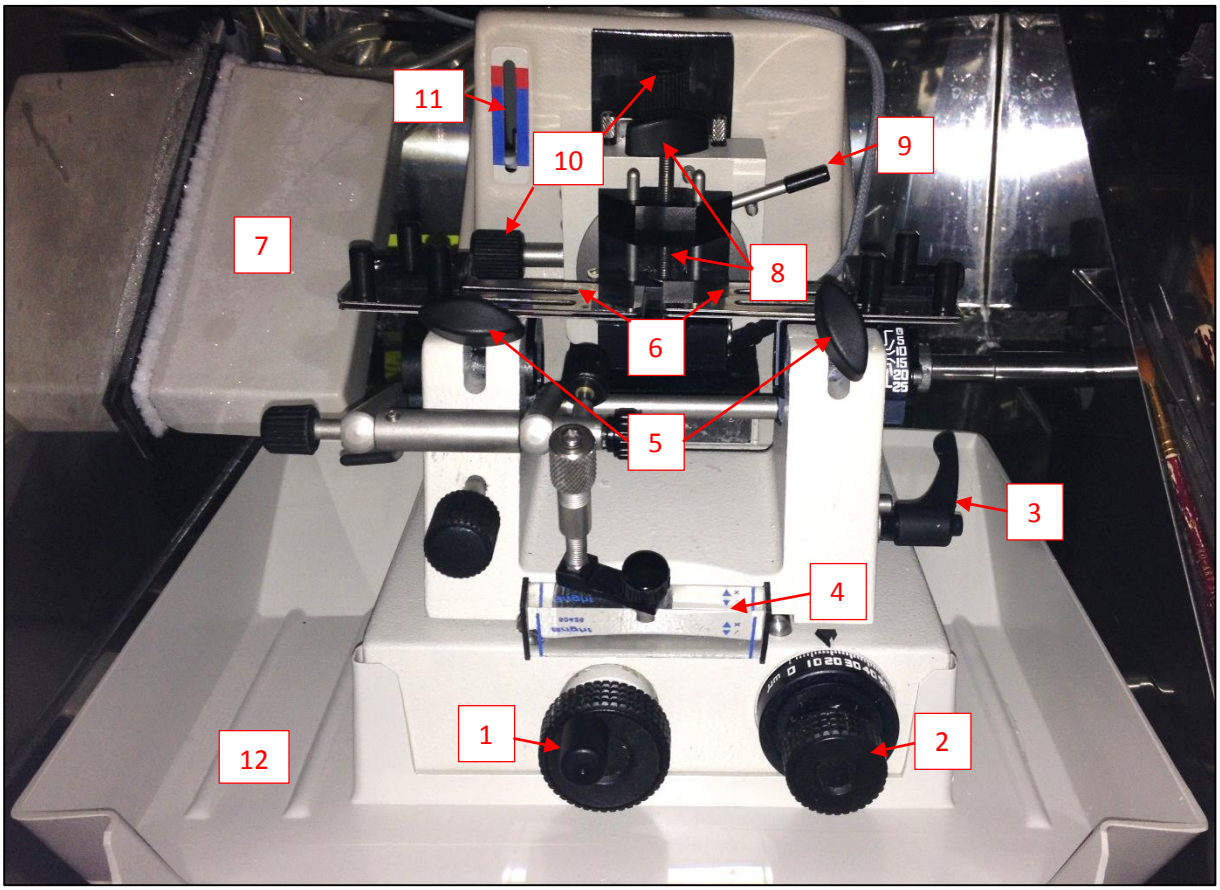


Figure 10: The cryostat components and controls. 1: Blade platform adjustment knob; 2: Section thickness adjustment knob; 3: Blade platform lock; 4: Anti roll guard; 5: Blade lock knobs; 6: Blade covers; 7: Quick freeze cover; 8: Chuck holder and tightening knob; 9: Chuck holder lock; 10: Chuck holder adjustment knobs; 11: Chuck holder distance marker; 12: Plastic collector.

c. Remove the quick freeze cover and place aside within the cryostat.
d. Place the frozen samples either on the quick freeze platform or within the -40°C freezer.
e. Close the sliding lid (Figure 11).

Figure 11: Closing the sliding lid on the cryostat.

f. Adjust the specimen and chamber temperature to -20°C by pressing “Set” and the arrows (Figure 12).

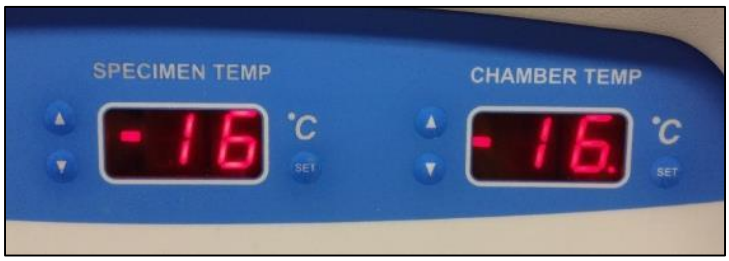


Figure 12: Specimen and chamber temperature controls.

g. Remove the cryostat blade from the freezer, unwrap it, and wipe with ethanol to clean off the WD40. DO NOT USE WATER TO CLEAN BLADE (Figure 13).

Figure 13: Blade removed from wrapping and cleaned with ethanol.

h. Lift the blade covers and place the blade into the crystat. Tighten the blade clamps, replace the covers, and place the anti-roll guard onto the blade (Figure 14).

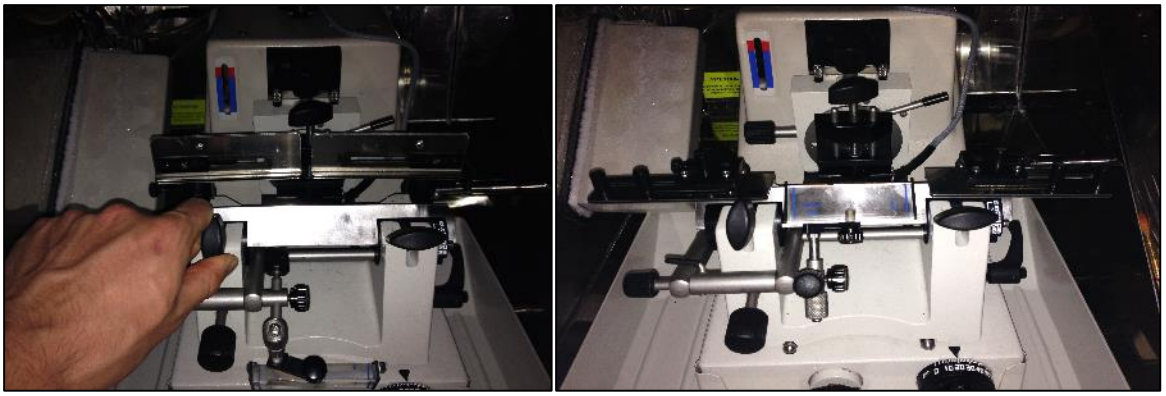


Figure 14: Placing the blade into the cryostat. Left, tightening the blade screws; Right, the finished blade setup.

i. Ensure the roll guard is lined up on the edge of the blade as shown in Figure 15.

Figure 15: The roll guard lined up on the edge of the blade.

Perform the following steps in low lighting to preserve the fluorescent cell tracker marker.

4. Prepare the samples for sectioning
   a. Locate a cryostat chuck and place a dab of OCT solution in the middle (Figure 16)

Figure 16: Cryostat chuck with OCT dab in the middle.

   b. Remove the embedded sample from its mold by peeling away one of the panels and place the top of the mold onto the OCT dab.
   c. Place the chuck on the quick freeze platform and keep it there until the sample freezes to the chuck.
   d. Pour more OCT solution around the edge of the sample to ensure the sample will adhere to the chuck during sectioning. See Figure 17 for a sample fully affixed to a chuck.
   e. Record which sample goes onto which chuck.
   f. Repeat for as many samples as possible.

Figure 17: Embedded BVM sample affixed to a cryostat chuck.

5. While waiting for samples to freeze to their chuck, label 3-4 Thermo Scientific Up-Rite slides per sample with the information contained on each sample's mold along with the sectioner's initials (Figure 18). Use pencil or Histoprep pen.

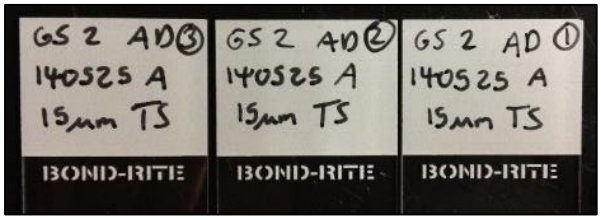


Figure 18: Slides labeled with the necessary BVM information.

6. There are two sectioning modes: manual and automatic. These modes are controlled by the sectioning handle on the right of the device. To manually section, pull out the lock knob and rotate the handle clockwise (Figure 19, left). To automatically section, bring the handle to 6 o'clock, push the lock knob back in place, and press the right foot pedal (Figure 19, center and right).

Figure 19: Manual vs automatic cryostat control. Left, automatic mode; Center, manual mode; Right, automatic foot pedal.

7. Place a finished chuck into the chuck holder of the cryostat and tighten the holder with the thumb screw above the chuck holder (Figure 20).
8. In manual section mode (Figure 19), raise the sample level with the blade.
9. Unlock the blade platform by pushing in the blade platform lock (Figure 10, #3) and move the platform toward the sample by rotating the Blade platform adjustment knob clockwise (Figure 10, #1). Stop when the blade is about 1 cm away from the sample face.
10. By moving the sample up and down in front of the blade, ensure the sample face is parallel to the blade edge. If not, adjust the sample face plane by first unlocking the holder (Figure 10, #9) and using

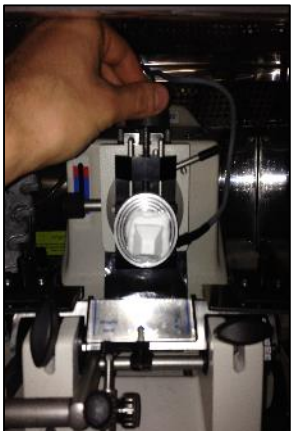


Figure 20: Chuck placed and tightened into holder.

the two adjustment knobs (Figure 10, #10): the left one for horizontal, and the top one for vertical alignment.

11. Once parallel, move the blade until it lightly touches the sample and lock the blade platform by pulling back on the blade platform lock.
12. Manually section the sample until the BVM can be seen (Figure 21).

Figure 21: The BVM visible in the embedded sample.

13. Switch to automatic mode (Figure 19).
14. Ensure the automatic sectioning limits are similar to those shown in Figure 22. Additionally, ensure the speed setting is slightly below the first line as shown.

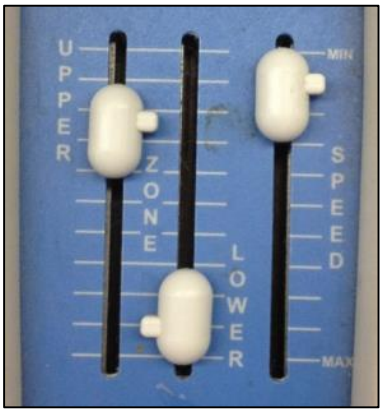


Figure 22: An example of the automatic sectioning settings.
Verify the speed setting is the same.

15. Press the right foot lever to begin automatic sectioning.
16. If the upper and lower limits for automatic sectioning are incorrect, adjust them accordingly.
17. Once the automatic limits are correct, ensure the section thickness is 15µm (Figure 10, #2) and that the chuck holder and blade platform locks are locked.

18. To create a good quality section, first perform a fast manual section, brush the scrap off of the blade using a paintbrush from inside the cryostat, and then perform a slow automatic cut (Figure 23).

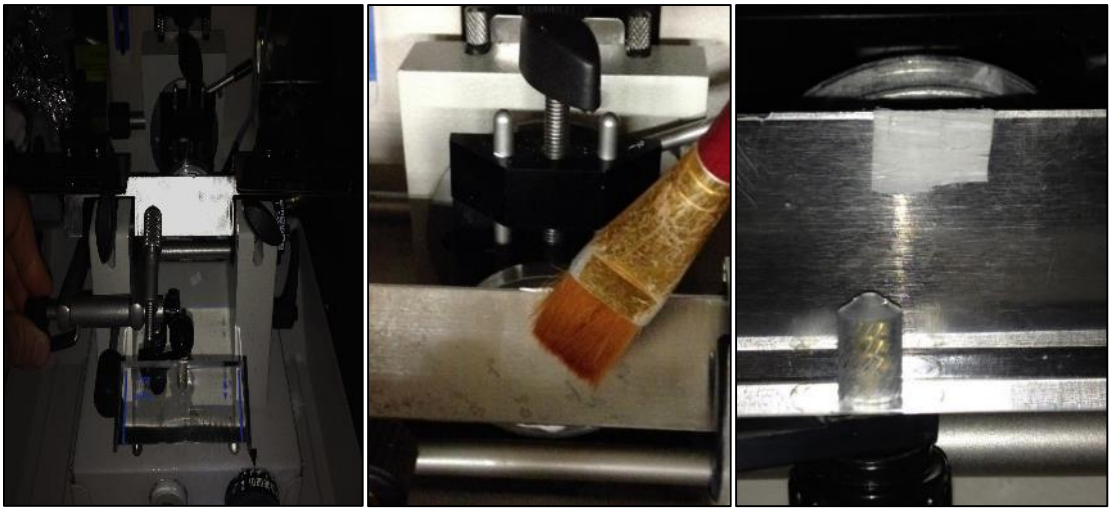


Figure 23: Performing a section. Left, the scrap from a fast manual section; Center, brushing the scrap off; Right, a good quality section.

19. If the sample is not sectioning well, either try cutting on a different part of the blade, lifting the roll guard slightly while cutting, moving the roll guard forward or backward, closing the clear lid while sectioning to keep the chamber temperature cold, or try a larger sectioning thickness.
20. Once a quality section is obtained, lift the roll guard to reveal the section and transfer it onto its corresponding slide.
    a. Start by placing the sections as close to the tab as possible and moving down the length of the slide.
    b. To transfer, use a quick rolling motion that allows the whole sample to touch the slide long enough to melt the sample to the slide but quick enough that it then does not freeze to the blade (Figure 24).

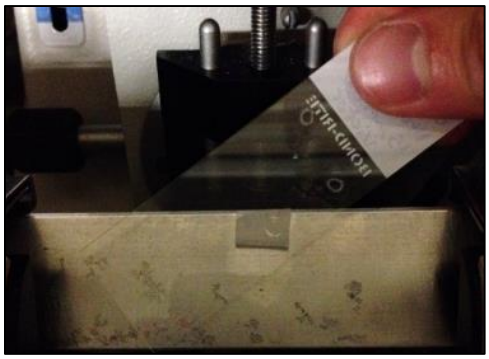


Figure 24: Transferring a sample onto a slide.

c. Try to fill up each slide with 6-10 sections (Figure 25). Record the section thickness on the completed slide and place into a slide box.

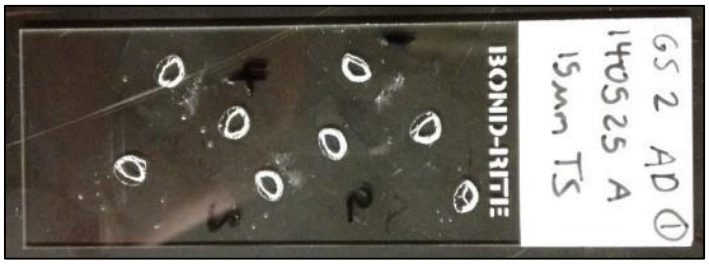


Figure 25: A completed slide containing four good quality sections.

21. Once 12-15 quality sections are obtained (usually 3 to 4 slides), remove the chuck from the holder and place outside for 1-1.5 minutes to let the OCT attached to the chuck soften.
22. Once soft, remove the sample from the chuck with the razor blade within the cryostat and place into its mold (Figure 26).

Figure 26: Removing samples from chuck for storage. Left, softened OCT solution; Right, removed Sample placed into its mold.

23. Re-wrap the mold in foil and place into the freezer for storage.
24. Repeat steps 5-22 for each sample. If the chuck holder distance marker ever gets close to the red region (Figure 10, #11), immediately stop sectioning, switch to automatic mode (Figure 19), and press the rewind button to retract the chuck holder (Figure 27). Then repeat steps 8-11 to reposition the blade.

Figure 27: The rewind button to retract the chuck holder.

25. Clean up the cryostat.
    a. With the cryostat in automatic mode, rewind the chuck holder (Figure 19 and 27)
    b. Retract the blade platform fully (see step 9).
    c. Remove the blade, spray with WD40, re-wrap in the blade paper, place inside the box, and place the box back into the freezer.
    d. Replace the quick freeze cover (Figure 10, #7).
    e. Remove the plastic collector (Figure 10, #12) surrounding the blade platform track and remove the cryosectioning waste with ethanol. Wipe clean with a paper towel or kimwipe. DO NOT CLEAN WITH WATER.
    f. Remove sectioning waste from the cryostat chamber with ethanol and a paper towel or kimwipe. DO NOT CLEAN WITH WATER.
    g. Replace the plastic collector.
    h. Replace the original lid. Verify the lid is fully shut (Figure 28).

Figure 28: The cryostat lid fully shut.

    i. Set the specimen and chamber temperatures back to -16°C (Figure 12).
    j. Clean the chucks with water and scrub brushes and dry thoroughly. Replace them into their storage positions.

G Hematoxylin and Eosin Staining Protocol

1. Turn on oven and set to 100°C.
2. Place slides into a staining rack (or multiple if necessary).
3. Once oven is ready, bake slides for 5 minutes.
4. While oven is heating up or slides are baking, prepare 15 staining dishes:
    a. 1x acetone
    b. 3x distilled water
    c. 1x hematoxylin
    d. 1x clarifier
    e. 1x blueing reagent
    f. 3x 95% ethanol
    g. 1x eosin
    h. 2x 100% ethanol
    i. 2x xylene
5. If 15 staining dishes are not available, you can reuse the distilled water dish filled with new water for each use.
6. Remove slides from oven and let cool in fume hood for 3 minutes.
7. Perform the staining as follows:
    a. 5 min – Acetone
    b. 3 min – Air dry in hood
    c. 5 min – Distilled water
    d. 3 min – Air dry in hood
    e. 2 sec – Hematoxylin
    f. 1 min – Distilled water
    g. 3 min – Air dry in hood
    h. 30 sec – Clarifier
    i. 1 min – Distilled water
    j. 3 min – Air dry in hood
    k. 30 sec – Blueing reagent
    l. 15 sec – 95% ethanol
    m. 3 min – Air dry in hood
    n. 3 sec – eosin
    o. 2x 15 sec – 95% ethanol
    p. 3 min – Air dry in hood
    q. 2x 15 sec – 100% ethanol
    r. 3 min – Air dry in hood
    s. 2x 4 min – Xylene
    t. 3 min – Air dry in hood
8. Apply coverslip.
9. Dry in fume hood for 24 hours.

H Compliance Tester Characterization Raw Data

Table H1: Raw Data Used to Calculate Percent Compliance

Lighting	Dark			Normal			Light		
Size	% ΔD	ΔP (psi)	%C (1/100mmHg	% ΔD	ΔP (psi)	%C (1/100mmHg	% ΔD	ΔP (psi)	%C (1/100mmHg
1/2	5.796	10.665	1.051	8.204	10.710	1.481	14.720	11.284	2.522
	5.148	7.690	1.294	6.728	10.303	1.263	12.624	10.345	2.360
	5.381	8.610	1.208	7.427	10.505	1.367	9.249	10.171	1.758
1/3	6.261	9.583	1.263	8.376	10.091	1.605	12.998	12.887	1.950
	6.714	8.784	1.478	7.714	7.359	2.027	10.542	9.828	2.074
	6.481	7.057	1.776	8.979	8.837	1.965	10.693	7.158	2.889
1/4	11.992	8.796	2.636	11.548	9.541	2.340	14.900	10.770	2.675
	10.098	9.588	2.037	13.071	10.519	2.403	14.748	7.600	3.752
	19.683	11.310	3.365	12.278	10.144	2.340	14.338	7.854	3.530

One-way ANOVA: normal, dark, normal, norm, normal, ligh, small, norma, ...

```
Source  DF      SS     MS     F      P
Factor   8  11.365  1.421  9.06  0.000
Error   18   2.822  0.157
Total   26  14.188

S = 0.3960   R-Sq = 80.11%   R-Sq(adj) = 71.27%

                                    Individual 95% CIs For Mean Based on
                                    Pooled StDev
Level             N    Mean   StDev   -+---------+---------+---------+--------
normal, dark      3  1.1869  0.1233   (-----*-----)
normal, normal    3  1.3715  0.0972     (-----*-----)
normal, light     3  2.2507  0.4444                (-----*-----)
small, normal     3  1.8473  0.2167           (-----*-----)
small, dark       3  1.4845  0.2882       (-----*-----)
small, light      3  2.2929  0.5220                (-----*-----)
smaller, dark     3  2.6563  0.6767                    (-----*-----)
smaller, normmal  3  2.3706  0.0390                 (-----*-----)
smaller, light    3  3.3409  0.5718                              (-----*-----)
                                     -+---------+---------+---------+--------
                                    0.80      1.60      2.40      3.20
Pooled StDev = 0.3960

Grouping Information Using Tukey Method
                  N    Mean  Grouping
smaller, light    3  3.3409  A
smaller, dark     3  2.6563  A B
smaller, normmal  3  2.3706  A B C
small, light      3  2.2929  A B C D
normal, light     3  2.2507  A B C D
small, normal     3  1.8473    B C D
small, dark       3  1.4845      C D
normal, normal    3  1.3715      C D
normal, dark      3  1.1869        D
Means that do not share a letter are significantly different.
```