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Development of an rQPLI-Compatible Translatable Stage for Imaging Collagen Gels

Lila Dickstein and Matthew Riak
Abstract

Tendons and ligaments are orthopedic tissues made up of collagen fibers and are responsible for attenuating load when a tensile force is applied. There are many different imaging modalities used to visualize collagen fibers, but each has limitations that preclude use in dynamic microstructural analysis of musculoskeletal soft tissues. Because of this, a technique called reflectance quantitative polarized light imaging (rQPLI) has been developed. However, as this technique is in development, ongoing work is being conducted in the Lake Lab at Washington University in St. Louis to understand how extracellular matrix properties affect polarization state of light to inform data interpretation from rQPLI. To perform experiments to this end, a translational stage needed to be fabricated to facilitate high throughput imaging of collagen gels with rQPLI. As detailed in this report, a literature search was performed to determine a suitable setup that could be fabricated in house and would be compatible with rQPLI. A viable setup was chosen that was based on modifications of a miniature computer numerical control (CNC) mill and previously used in high throughput imaging of crystals in 96-well plates. This report details the fabrication process of the system and its validation for use with rQPLI.
Introduction

Musculoskeletal soft tissues such as tendons and ligaments are fibrous, collagenous tissues in the body which either connect muscle to bone or sets of bones together, respectively. Injuries to these tissues are extremely prevalent in the world - over 1.7 billion people suffer from these injuries annually. [1] Tendons and ligaments are made up of aligned collagen fibers which are responsible for much of the force attenuation when a tensile load is applied. [2] Because collagen structuring is so important to overall mechanics in tendons and ligaments, it is important to be able to visualize the collagen architecture to better understand tissue function. Although there are many types of imaging modalities currently in place that are capable of visualizing collagen (i.e. magnetic resonance imaging (MRI), computed tomography (CT), optical coherence tomography (OCT), etc.), they each have limitations in areas such as spatial resolution, tissue processing requirements, and a limited field of view. These limitations preclude their use in dynamic, whole tissue orthopedic imaging applications.

To combat these issues, a technique called reflectance quantitative polarized light imaging (rQPLI) has been developed. [3] Tendons and ligaments have birefringent properties, meaning that they can polarize incident light in proportion to their structural anisotropy. [2] This property is leveraged in rQPLI to obtain dynamic, quantitative measurements of the alignment of collagen fibers in real time. As rQPLI is still in development, there are several questions remaining as to how different extracellular matrix (ECM) properties besides collagen fiber anisotropy affect how polarized light is transmitted and reflected from tendons and ligaments. Some ECM properties that are of particular interest to orthopedic applications that are hypothesized to influence polarization are collagen density, crosslinking, and biomineralization. When light interacts with scatterers within tissue or with birefringent material, it changes its polarization state. [3] Studying how light
polarization is affected by these properties of collagenous tissues can help create a better fundamental understanding of polarized light-tissue interactions in a clinically relevant context. Further, complete characterization of rQPLI can help develop knowledge of tendon and ligament structure-function relationships to better inform treatments of common but poorly understood soft tissue injuries.

Our long-term objective is to test, in a piecewise manner, how individual ECM properties affect the polarization state of light. Using a tunable collagen gel platform, we aim to create gels isolating a specific property of interest, and subsequently image each with rQPLI. To perform this imaging in a high throughput manner, an imaging setup is necessary to take focused images in a repeatable manner. These images could then be analyzed to help better understand ECM properties’ effect on polarized light. Therefore, our objective for this semester was to build an imaging setup that can accomplish this. An automated microscope (AMi) setup based off a mini CNC machine was created to facilitate the imaging process of collagen gels. [4] This setup is designed to hold a 24 well plate, but other size well plates or sample vessels can be used as well. The machine allows the sample of interest to be moved in all three dimensions with fine precision, which ensures that focused and detailed sample images can be taken. Finally, we confirmed the imaging setup’s compatibility with rQPLI.

Methods

*Literature Search.* A thorough literature search was first conducted to identify a candidate for an imaging system that would be fabricated over the course of the semester. The AMi system was identified as it is an inexpensive translatable stage that has been used as an automated microscope. [4] It uses the basic structure of a miniature CNC machine with a Python-based graphical user interface (GUI) to achieve this automated functionality. The AMi integrates a Raspberry Pi camera
so that the system is fully contained. [4] It has been used to search for protein crystals in 96 well plates. Most of the assembly was the same as described in the instruction manual that came with the CNC machine, with a few alterations. [4] Figure 1 shows the schematic of the original AMi system, an image taken of a crystal in a 96 well plate using the original AMi, the CNC machine kit the AMi is modified from, and the fully assembled and modified AMi system to be used in conjunction with rQPLI for high throughput imaging of collagen gels (AMi-rQPLI).

The user interface of the AMi system is programmable so that the stage can automatically move to capture an image of each well in the well plate. There are previous publications describing both the original system [5], and a less expensive device that serves the same basic functions but without a fully integrated camera component. [6] This operation software can be modified to be used with rQPLI. The premise is the same, imaging samples in well plates. The primary use for

![Image](image1.jpg)

**Figure 1:** (A) Original Automated Microscope (AMi) system [5] (B) Image taken of crystals with original AMi setup [5], (C) CNC machine kit that was modified to create AMi device, (D) final modified AMi-rQPLI.
the AMi-rQPLI system will be to image and analyze collagen samples in 24 well plates. As such, the polarization camera will not be integrated into the system, meaning that the code for device operation need not involve control of the camera. However, the design must include room for an externally controlled light source and polarization camera, as these are the main components of rQPLI. [7] 

Hardware. The first step in the build process was to make a list of parts required for assembly. [5] This compiled list is represented in Table 1.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Quantity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1610 CNC machine kit</td>
<td>1</td>
<td>Forms basis of AMi-rQPLI system[4]</td>
</tr>
<tr>
<td>HDMI monitor</td>
<td>1</td>
<td>Monitor for Raspberry Pi</td>
</tr>
<tr>
<td>Dupont jumper wire 20CM package</td>
<td>20</td>
<td>Connected wires from limit switches to Arduino</td>
</tr>
<tr>
<td>Raspberry Pi 3 B+</td>
<td>1</td>
<td>Connected to Arduino and monitor. Displays GUI on monitor</td>
</tr>
<tr>
<td>Heat shrink tubing</td>
<td>20</td>
<td>Covered wire connections</td>
</tr>
<tr>
<td>Solder machine and solder</td>
<td>1</td>
<td>Connected Arduino/Raspberry Pi wires</td>
</tr>
<tr>
<td>Keyboard and mouse</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>Wood board</td>
<td>1</td>
<td>Used as base (1ft x 2ft)</td>
</tr>
<tr>
<td>End switches (CYT1073)</td>
<td>6</td>
<td>Used as limits for movement of machine</td>
</tr>
<tr>
<td>12 V DC power supply (2 A)</td>
<td>1</td>
<td>Power source for Raspberry Pi</td>
</tr>
<tr>
<td>Electrical tape</td>
<td>1 roll</td>
<td>Covered connections between wires</td>
</tr>
<tr>
<td>Wood screws</td>
<td>6</td>
<td>Attached frame to base board</td>
</tr>
<tr>
<td>PLA</td>
<td>1 roll</td>
<td>Material used for 3D printed parts</td>
</tr>
</tbody>
</table>

Table 1: List of equipment needed for AMi-rQPLI assembly.
The key differences between the original and modified CNC machines are that the y-axis goes below the y-base, and that the frame holding the x- and z-axes is placed in the middle of the gantry, instead of the back. [4] The finished base structure is shown in Figure 2A.

Once the basic frame was assembled, the Arduino included in the CNC kit was added to the frame’s gantry, shown in Figure 2B. The wires for each of the three motors were connected to the Arduino in their respective spots (Figure 2B). Limit switches were glued to each side of the frame to make sure the machine did not move too far in one direction. Wires were connected between the limit switches and the Arduino in their respective positions. To extend the wires, jumper wires were soldered together, and heat shrunk was used to cover the connections. A 1”
thick wooden board was used for the base of the machine. Other materials, such as marble and acrylic, are also suitable. Wood was chosen for ease of cutting and drilling, and low cost. The frame was attached to the base using wood screws (Figure 2D). A part was designed in AutoDesk Fusion 360 and 3D printed to attach to the gantry and hold the well plate (Figure 2C). The complete hardware assembly can be seen in Figure 1D.

Software. The Raspbian operating system was imaged onto the Raspberry Pi that is used for control of the AMi-rQPLI. Preexisting software that was developed for operation of the original AMi was downloaded and transferred to the Raspberry Pi. [4] This software contained two major functionalities: (1) a parent code that initialized all hardware and opened communication with the AMi-rQPLI’s Arduino and (2) a GUI called bCNC that is used for operation of the frame in three dimensions. [6, 8 All files were placed in the same directory on the Raspberry Pi (i.e., Desktop) for proper operation. Initiation of the parent software and the GUI was performed from the command line. Figure 3 depicts the GUI opened on a monitor.

A procedure was written for how to run the machine and contains images of the setup, GUI as well as troubleshooting steps (Appendix A1).

Figure 3: GUI opened on monitor showing position of machine and arrows used to move machine
Validation of AMi-rQPLI. To ensure the frame was operational, the GUI was opened and movement in all directions was tested. Occasionally the machine would get stuck, in which case the base board was sanded down so there was no friction with the frame. Additionally, it was necessary to add WD40 to make the machine’s movement smoother. As previously mentioned, the machine was designed to allow for a controlled light source and polarization camera which are used in rQPLI.

Results & Discussion

Hardware. There were a few deviations from the original AMi system that were required to ensure the hardware’s compatibility with rQPLI. One decision we had to make was which material to use for the machine’s base. Articles described various materials that could be used [6], but we decided to use wood because it is durable, inexpensive, and easy to use.

While screwing down the machine to the board, occasionally the machine would become stuck because there were issues with the amount of torque being applied to the metal rods which run along the bottom of the machine (Figure 2D). To work around this, we decided to decrease the amount of torque in the screws to relieve some of the stress. Additionally, we had to sand down part of the wood to allow the machine to move more smoothly, as it would occasionally get stuck and run into the base. Furthermore, WD40 was used to facilitate motion.

Finally, cable management proved to be an issue during the frame fabrication process as depicted in Figure 4. The wires needed to be kept away from the moving parts so they would not get caught. To keep them organized, they were taped down using electrical tape and connections were covered with heat shrink tubing or electrical tape to minimize the possibility of injury.
Software. One of our biggest challenges was proper operation of the bCNC GUI which was used to control the machine’s movement. The proper python code was all downloaded onto the Raspberry Pi, but the interface would not launch. We ameliorated this problem by finding a Universal G-Code Sender on GitHub. Downloading this code onto the Raspberry Pi integrated all aspects of the software, allowing the bCNC GUI to launch and operate the AMi-rQPLI.

The final step was integrating limit switches with the software. Six limit switches were originally added (2 for each movement direction), but we were only able to use one in each direction. The Arduino did not work when multiple limit switches were used in the same direction. It is possible that this issue is caused by the fact that the Arduino does not know which limit switch to use, so it disregards both switches. In the future, we plan change the wiring schema and conduct more tests to see if limits in both directions on each axis will function correctly. We tried changing the GUI settings to add soft limits but will explore this feature further in future work.

Validation. To ensure the design’s validity, the first step was to test movement in all axes and determine whether the limit switches worked correctly. This was done by moving the machine in all axes and making sure it did not stop. Step sizes range from as small as 0.001 to larger than 100 mm at a time. The x and y axes can move a few hundred mm, while the z axis can move less

Figure 4: CNC machine with completed and unsorted wires.
than 50 mm. Next, we tested our ability to image a sample using the AMi-rQPLI system. An iPhone was used to image pieces of paper dropped into the wells in place of collagen fiber samples, which is shown in Figure 5. Once focused and accurate images were obtained, it was clear that the camera setup would work with real samples.

**Figure 5:** Completed CNC machine with iPhone camera placed above well plate to take images of test samples.

**Conclusions and Future Directions**

This automated microscope (AMi-rQPLI) system is now fully built and ready to be used for high throughput testing of collagen samples in well plates. The next step for this project is to develop and validate a testing protocol for rQPLI of different collagen gels in a well plate. This translatable stage can be used for efficient testing of gel samples with different ECM phenotypes, which will allow us to both develop and carry out this protocol as quickly as possible.

While this stage is fully usable, there are some future steps that can be taken to maximize its functionality. In regard to hardware, the wooden base can be coated with epoxy to prevent damage due to moisture or spillage. Another option is to switch the material of the base to something more durable, such as marble. One of the aforementioned issues that we ran into, is that we had trouble getting all limit switches functioning at once. This is not a necessary feature, but it would be nice to have them working if a user
accidentally tries to send it past its limit, it will prevent wearing down the lead screw. Lastly, another helpful feature within the code would be a program that automatically moves to each well so that the user can image the entire well plate with the click of one button. It would start with the camera aligned with a designated corner of the well plate, then move the gantry to each well with a pause in between so that the user can take a photo. These modifications are not integral to the use of the AMi but will all aid in its use for analysis of rQPLI testing of various ECM properties in collagen hydrogel samples.
References:


Appendix

A1. Operation Protocol for AMi-rQPLI

The following is a protocol written up describing how to use the automated CNC microscope. Included are images depicting the setup and the GUI, along with troubleshooting steps.

Procedure:
1. Plug in Raspberry Pi to wall outlet
2. Monitor should turn on (connected to Pi)
3. Open Terminal (located at top left of screen) - logo shown in figure 1
4. Type (in Terminal):
   a. cd Desktop/AMi (click enter)
   b. python3 AMiGUI.py (click enter)
      i. Messages appear in terminal, you may disregard these
5. Plug in arduino to wall
6. Press ‘On’ button on Arduino - fan on Arduino should start when Arduino is on, shown in figure 2 (white button)
7. Type (in Terminal):
   a. python -m bCNC (click enter) *(should open GUI interface)*
8. If the control section is not selected (at top of GUI) click control section. GUI should look like figure 3
9. Control gantry using arrows in bottom left (can set how far it moves) (see figure 3)
   a. Everything should be done under ‘Control’ tab of the interface
   b. Labeled axes are shown in figure 4
   c. Note that for z-axis, directions are flipped so up arrow will move the gantry down, and vice versa
   d. NOTE: Do not use ‘Move Gantry’ button
      i. This may try to send gantry out of range of machine
   e. Gantry movement can be tuned by clicking down arrow on bottom of screen, shown in figure 5. *(The 5.0 shown below)*
      i. Don’t set Z-rate to more than 50.
10. When finished, close terminal, click ‘off’ button on Arduino and unplug devices.
**Figures**

*Figure 1: terminal button*

*Figure 2: On/Off button on Arduino*
Figure 3: GUI setup
Figure 4: Labeled axes on gantry

Figure 5: Step size adjustment for gantry movement
**Troubleshooting:**

- If an Alarm shows up in the status bar, click reset, wait a few seconds and then click unlock, which should allow gantry to move again. The status bar should be updated to idle. If not, repeat this step. This occasionally happens two times in a row.
- Click arrow and sounds like motor is moving but gantry doesn’t move (usually happens with y-axis)
  - This means that the lead screw has come out of the motor. Unscrew it then screw it back in
- If the gantry runs into an edge without stopping click stop button at top of screen