Construction and Performance Evaluation of a Confocal Laser Scanning Microscope

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Abstract

We introduce the motivation and principles of a confocal laser scanning microscope (CLSM). We then present and explain the operation of our CLSM setup which is distinct in its use of readily available and 3D printed components. The setup utilises standard light microscopy objectives, a 3D printed open-source high precision delta stage from the OpenFlexure project as well as an Arduino microcontroller and a Raspberry Pi microprocessor. We discuss various issues we encountered during the construction as well as the solutions we have come up with. The operation of a program which analyses the data in the form of images to produce z-scan curves is explained. We present our measurement of the thickness of PVC electrical tape to be 137 ± 17 μm in good agreement with the value obtained using a screw gauge micrometer. At the end we discuss further improvements which should be sufficient to enable the setup to perform automated 3D surface scans.

I. Background

The aim of the project was to construct a simple CLSM setup with the use of standard opto-mechanical components as well as readily available electronics and 3D printing.

The initial inspiration for the project was a video by the YouTube channel ”Breaking Taps”, which presents the construction of a home-built vertically oriented CLSM setup as well as final 3D surface maps of samples such printed circuit board (PCB) traces.

The initial goals were:

- Construct a working CLSM setup and use it to collect data for a series of manual z-scans to determine the thickness of a control sample which can be measured using a screw gauge micrometer.
- Write a data analysis program in Python which can take the data, in form of RGB images, and calculate the average intensity over the image sensor. Then plot the intensities against displacement in the *z-axis*, determine the shift of the peaks to calculate the thickness of the control sample.
- Construct a motorised 3D translation stage capable of sub-micron positioning, which can be used to automate the z-scans.
- Automate the z-scans by synchronising the intensity measurements with the positioning of the sample using a Raspberry Pi Microprocessor and peripheral electronics.

The extended goals were:

- Perform a full 3D surface scan, comprised of z-scans at different x and y coordinates, for an interesting sample such as the surface of a 2p coin, and analyse the data to reproduce a 3D model of the surface.
- Try to obtain an atomic force microscopy (AFM) surface scan which can be compared to our scan and used to evaluate the performance of our setup.

II. Description of Project Work

A. Project Initiation

The work commenced on the 16$^{\text{th}}$ of May, initially meeting our supervisor, Chiara Labanti (CL), on the 19$^{\text{th}}$ to discuss the initial project idea and risk assessment. We continued having weekly meetings with CL and sent regular updates in the form of photos and messages via Microsoft Teams. The final risk assessment was completed by LR and LK and approved on the 25th of May. In the meantime, MH inquired about the availability of opto-mechanical components and microprocessors.

B. Optical Setup

On the 25th of May with TG and LR, LK led the construction of the confocal optical setup.

For the objective, both a modified microscope eyepiece system as well as microscope objectives with different magnifications ranging from $4\times$ up to $60\times$ were tested. It was found that the greater magnification objectives gave narrower intensity peaks and therefore higher accuracy, however, this was a trade-off for a lower range and a much smaller focal length. Mounting the microscope objectives required us to make an adapter which allows the tip of the objective to be near the sample surface without obstructing the movement of the stage.

LK and LR tested the optical setup on the 6th of June by bringing a white card into focus and observing the reflected light intensity. We encountered an issue with a constant background intensity due to light reflected from the surfaces of the optical elements, especially the beam splitter. We attempted to mitigate this by purposefully misaligning the optics to spatially separate the unwanted spot so that we could position the pinhole to block it out. On the 7th of June, LK with the help of LR and TG have built a light-proof enclosure made out of black foam board. This was done to eliminate the effects of varying background light intensity.

C. Preliminary Data Collection

During the week starting on the 6th of June, preliminary z-scans have been carried out using a manual linear translation stage varied in increments of $50\ \mu\mathrm{m}$. At first, LK and TG used a silicon (Si) photodiode with a transimpedance amplifier connected to a precision voltmeter. The photodiode was later replaced by a ThorLabs CMOS camera in order to decrease the time taken for measurements. Later during the week, LR and LK performed multiple z-scans of an increasing number of layers of PVC electrical tape. Initially LK had the idea to use kapton tape which is not as elastic as the PVC tape and therefore has a more consistent thickness. However, the tape is semi-transparent giving two peaks due to reflection at both the top and bottom surfaces.

D. Data Analysis

After the collection of preliminary data, LR and LK have worked on an analysis program in Python which uses the OpenCV library to analyse each image and convert it into an average intensity value over the whole sensor. In order to do this accurately, the relative luminosity of each of the RGB colours had to be accounted for as well as the RGGB Bayer filter in the Raspberry Pi camera module.

E. 3D Translation Stage

Later during the lab session on the 24th of June, we discussed various methods for automated 3D positioning of our sample. These methods included motorised linear translation stages, piezoelectric stacks, the delta stage (DS) from the OpenFlexure project, and even attempting to motorise a manual multi-axis linear translation stage assembly. After investigating each option, we concluded the delta stage to be the most viable solution. In practice, motorising a manual multi-axis stage would be difficult due to the motion in one axis displacing the other axis requiring the motors to move with the stage. The other options would rely on stacking multiple linear stages perpendicular to each other making the alignment of axis difficult.

MH has been assigned to lead the construction of the DS with the help of TG. Ivan Hermida Estornell 3D printed the DS in 2 days using PLA filament with PVA supports. They encountered issues such as: failure of flexure mechanisms, incomplete dissolution of the support structures, and the use of a heat gun in proximity to the DS causing the bottom end to deform. Overall, we ended up reprinting the stage twice.

To mount the sample onto the stage, LR and TG obtained a small rotation stage with a 3D printed sample mount on the top. LK then designed and laser cut adaptor plates to mount the rotation stage onto the DS and then the whole DS onto a linear translation stage which can be used for calibration.

F. Control and Electronics

The Raspberry Pi and Arduino interfacing was managed by MH. Initially, using the Raspberry Pi's available in the lab (Model 2 B, and Model 3 B), we found that neither could handle the minimum system requirements needed for the custom version of Raspbian we would need to control the DS. Subsequently, with a Raspberry Pi Model 4 B, we could test the viability of synchronising the DS with capturing images using the ThorLabs camera, or a photodiode arrangement. After attempts by MH to install the Linux SDK drivers as well as several proprietary Python libraries, it was impractical to attempt to synchronise the capturing of images due to latency through the Raspberry Pis USB port. In the end, a v2 Raspberry Pi CMOS camera module with a removed lens was used to capture the images. A server was set up to control the microscope remotely for long scans. The mounting and wiring of the electronics has been done by LK with the help of LR who soldered the connections for the stepper motor drivers.

G. Automated Scanning

From the 13^{th} of June, all of the efforts have been concentrated on getting the automated scanning working. We have encountered multiple issues, the largest one of which has been the auto-exposure adjustment which can not be disabled in the OpenFlexure software. This issue resulted in the breaking-up of the peak at high intensity values. We also encountered problems with the delta stage losing steps due to the stepper motors being unable to overcome the large resistance of the actuators. This has been solved by lubricating the gears, washers, and lead screws. Additionally, axial bearings and thinner O-rings could be used to further reduce the resistance from the actuators.

III. Summary of Results

Based on the analysis of the preliminary data, we have managed to determine the average thickness of PVC electrical tape to be 137 ± 17 μm. This can be compared to the 150 ± 10 μm measured using a screw gauge micrometer and the results can be seen to agree well with a percentage difference of 8.67%. It should be noted that the measurements obtained using the CLSM setup are expected to be slightly lower due to the sample mounting procedure which can contribute to stretching the tape making it thinner. Additionally, the measurement from the CLSM is an average of 3 layers of the same PVC electrical tape which has likely been stretched to different extent. This results in the standard error of the mean being an overestimate of the uncertainty of the CLSM setup. Based on the width of the produced peaks, we expect the actual precision to be of the order of micrometers.

IV. Conclusion

We have managed to accomplish all of the initial goals including the final automation of the z-scans. We have managed to show our CLSM setup to function as expected and furthermore, to be capable of measuring height with a micrometer level precision all while using a 3D printed stage.

Additionally, we have managed to collect data by performing automated z-scans, however, the analysis of the data has shown the intensity measurements taken with the v2 Raspberry Pi camera module to break down at greater intensities making it difficult to determine the position of the peak. In the last few days of the project, we managed to achieve noticeable improvements in the z-scans by fine-tuning the optical setup and the data collection software settings.

Future improvements to the setup would include replacing the DS with a 3D printed block stage which uses a different arrangement making it more suitable for our setup. Another improvement would be controlling the stage through a Python library and synchronising it with a different detector such as a CCD camera or a photodiode and an ADC.

V. Equipment Used and Purchased

The following items have been purchased:

• 1 Pack of 25mm M3 Hex Bolts	£6.00
• 1 Pack of 30mm ID, 34mm OD, 2mm CS, Nitrile O-Rings	£6.90
• 3 A1 Sheets of Black Foamboard	£25.50
• 2m Ribbon Cable for Raspberry Pi Camera (Had Connection Issues)	£5.20
• 1 Pack of Ribbon Cables of Assorted Lengths for Raspberry Pi Camera	£17.98
• v2 Raspberry Pi Camera Module	£24.90
• Raspberry Pi Model 4 B 2GB RAM (Minimum System Requirements)	£72.00
• 1 Pack of 5 28BYJ-48 Stepper Motors with ULN2003 Drivers	£33.98
Total price including VAT and excluding delivery:	£192.45

The following items have been borrowed or were already in our possession:

- 1 ThorLabs Nexus Optical Table
- 1 ThorLabs DCC1545M-GL CMOS Camera
- 1 ThorLabs CCM1-BS013/M Beam Splitter
- 1 ThorLabs MT Linear Translation Stage
- 1 ThorLabs XR Linear Translation Stage with Micrometer
- 1 ThorLabs PR Rotation Stage
- 1 ThorLabs Polarising Filter in a Rotation Mount
- 6 ThorLabs Optical Construction Rails
- 9 ThorLabs Post Holders with Base Plates
- 2 ThorLabs Compact V mounts
- 1 ThorLabs 4.5mW Class 3R Laser Diode (CPS532)
- 1 ThorLabs 5V Phono Jack Laser Diode Power Supply
- 1 ThorLabs LMR Fixed Lens Mount
- 3 ThorLabs SM Lens Tubes of Different Length
- 3 ThorLabs SM Lens Tube Mounts
- 1 ThorLabs SM Laser Diode Mount
- 1 ThorLabs Plano Convex Lens f=25mm
- 1 Generic Linear Polarisation Filter
- 5 Microscope Objectives with Magnifications: $4\times, 10\times, 20\times, 40\times, 60\times$
- 1 Generic 10x Microscope Eyepiece with f=18.5mm
- 1 Planoconvex Lens f=160mmm
- 1 Convex Lens f=100mm
- 3 Generic Filter Holders on a Post
- 1 Generic Pinhole
- 1 Agilent 34401A 6.5 Digit Multimeter
- 1 Generic Si Photodiode With a Built in Transimpedance Amplifier
- 1 Photodiode Mount
- 1 Aim TTi EL302R 30V 2A Linear Power Supply
- 1 Arduino Uno Wi-Fi Rev 2
- 1 Raspberry Pi Model 2 B, 512MB RAM (Insufficient RAM)
- 1 Micro SD Card with SD Card Adapter
- 4 Rubber Feet with Adhesive
- 1 Roll of PVC Electrical Tape
- 1 Roll of Kapton Tape
- Electrical Hardware: Wires, 5mm Jacks, Headers, Silicone Tubing, Heat Shrink Tubing
- Mounting Hardware: (M3, M4, M6) Screws, Nuts, Washers and Standoffs

The following items have been 3D printed or laser cut:

- 3 Delta Stage Main Body
- 1 Delta Stage Platform
- 4 Sets of O-Ring Retainers
- 1 Sample Mount
- 1 Adaptor Plate to mount rotation stage onto the delta stage
- 2 Adaptor Plate to mount delta stage onto the linear translation stage
- 1 Mounting Plate to mount delta stage directly onto optical table
- 1 Mounting Plate to mount electronics onto optical table

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VI. BIBLIOGRAPHY

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Article explaining the details of the delta stage and its use in light microscopy. This was used to understand the working principles of the delta stage and its initial intended use.
3) Q. Meng, K. Harrington, J. Stirling, and R. Bowman, The OpenFlexure Block Stage: sub-100 nm fibre alignment with a monolithic plastic flexure stage, Optics Express, vol. 28, no. 4, pp. 47634772, 2020.
Article describing the working principles of a 3D printed flexure mechanism block stage as well as evaluating its performance. This was used to explore possible alternatives to the delta stage towards the end of the project as well as to see the parameters of 3D printed stages utilising flexure mechanisms.
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Article about applications of CLSM in the field of material science and applied sciences. It was used to learn more about typical applications in material science and the surfaces which can be scanned. This article was also helpful in introducing the basic principles behind CLSM.
6) The ThorLabs website Available: https://www.thorlabs.com and the available product documentation has also been very useful in getting to know optomechanical components and their technical properties.
7) A. Mordvintsev and K. Abid, Opencv-python tutorials documentation, Available: https://docs.opencv.org/4.x/ and also on Google Scholar as OpenCV-Python tutorials documentation. The OpenCV Python library has been used for image processing in the data analysis program. To learn more about implementing this library we have studied the OpenCV Python library documentation and tutorials.

8) Texas Instruments Analogue Engineer's Circuit: transimpedance amplifier circuit Available: https://www.ti.com/lit/an/sboa268a/sboa268a.pdf?ts=1655907060912&ref_url =https%253A%252F%252Fwww.google.com%252F Has been useful to find out more about design of transimpedance amplifiers during the early stages when we were considering measuring intensities using a photodiode rather than a CMOS detector.
9) The code flashed onto the Arduino Uno to control the stepper motors has been taken from the gitlab repository Available: https://gitlab.com/bath_open_instrumentation_group/sangaboard/-/blob/master/arduino_code/README.md
10) The OpenFlexure Project website Available: https://openflexure.org/projects/deltastage/ Was used to find some preliminary information about the performance of the stage. More importantly, it has been used to obtain the delta stage files for the 3D printing as well as assembly instructions.
11) The OpenFlexure software and API Documentation for the control of the DS has been found a website Available: https://openflexure-microscope-software.readthedocs.io/en/master/basestage.html
12) The OpenFlexure Python library project which we wanted to use for controlling the DS while taking intensity measurements with a ThorLabs camera or a photodiode can be found here Available: https://openflexure-microscope-software.readthedocs.io/en/master/basestage.html
13) The Windows SDK files which MH was attempting to use for interfacing the ThorLabs CMOS or CCD cameras with the Raspberry Pi microprocessor can be found on the official ThorLabs website Available: https://www.thorlabs.com/software_pages/ViewSoftwarePage.cfm?Code=ThorCam
14) OpenFlexure build instructions Available: https://build.openflexure.org/openflexure-microscope/v6.1.5/docs/#/6_motor_controllers Has been used to understand the circuit required for the Arduino to drive the stepper motors.