

Development of a Home-Built Light Sheet Fluorescence Microscope for Cleared Tissue Imaging

by

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**Development of a Home-Built Light Sheet Fluorescence Microscope for
Cleared Tissue Imaging**

Koç University

Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a master's thesis by

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and have found that it is complete and satisfactory in all respects,
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examining committee have been made.

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Date: _____



To my family...
To other loved ones...
and
To you...

ABSTRACT

Light sheet fluorescence microscopy has become a popular technique for life sciences such as biomedical studies due to its advantages compared to confocal and epifluorescence microscopy. LSFM is a non-destructive technique using a narrow line of light; instead of a focused spot, for the illumination and it allows fast imaging with large field of view and reduced photobleaching effect.

In this study, we present development of a home-built LSFM for cleared tissue imaging set-up with commercially available equipment. LSFM involves formation of a thin sheet of laser light using a cylindrical lens. The sample is scanned along the light sheet; thus the sample planes are selectively illuminated. The selective illumination of the sample allows for selective fluorescent excitation at desired focal planes. Besides three-dimensional image of the sample is acquired by the reconstruction of selected planes.

The LSFM has been built utilizing Open Access platform for Selective Plane Illumination Microscopy. The sample was a stained cleared tissue with fluorescent tagged secondary antibodies. In order to illuminate the sample, several laser sources with a wavelength of 525 nm was used for example a Coherent Chameleon tunable laser with an SHG and a diode laser. Three-dimensional images were reconstructed from the imaged planes using the Fiji ImageJ software.

ÖZETÇE

Işık tabakası floresans mikroskopisi, konfokal mikroskop ve yansıma floresans mikroskop yöntemlerine nazaran çeşitli yararları nedeniyle biyomedikal alanı gibi yaşam bilimlerinde sıkça uygulanan bir görüntüleme yöntemi haline gelmiştir. LSFM yöntemi aydınlatma için odaklanmış bir nokta yerine dar bir ışık çizgisi kullanıldığından tahribatsız bir tekniktir bu da geniş görüş alanlı hızlı görüntüleme sağlamaktadır ve fotoışıldama bozulması etkisi de azalır.

Bu çalışmada ticari olarak erişilebilir donanımlar kullanarak kendi labaratuvarımızda yaptığımız LSFM doku götüntüleme düzeneğinin gelişiminini tanıtıyoruz. LSFM için silindirik mercek kullanarak ince bir lazer ışığı tabakası oluşturmak gerekir. Örnek bu ışık tabakası boyunca taranır böylece örnek düzlemleri seçime bağlı olarak aydınlatılır. Örnek düzlemlerinin seçime bağlı olarak aydınlatılması, istenilen odak düzlemlerinde tercihe bağlı floresan uyarılmasına imkan verir. Örneğin üç boyutlu görüntüsü bu seçili düzlemler kullanılarak elde edilir.

LSFM düzeneği "Open Access Platform for Selective Plane Illumination Microscopy" isimli açık kaynak platformundan yararlanılarak yapılmıştır. Görüntüleme için kullandığımız örnek, floresan boya ile boyanmış şeffaflaştırılmış dokudur. Örneği dalga boyu 525 nm olan birkaç lazer kaynağı ile aydınlatabilmek için SHG yöntemiyle Coherent Chameleon ayarlanabilir lazer ve diyot lazeri kullanılmıştır. Üç boyutlu görüntüler ise Fiji ImageJ yazılımı vasıtasıyla daha önce görüntülenen düzlemlerin birleştirilmesiyle oluşturulmuştur.

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NOMENCLATURE

ADC : Analog to Digital Converter

AR : Anti Reflection

DSLM : Digitally Scanned Light sheet Microscope

EFL : Effective Focal Length

ERS : Electronic Rolling Shutter

FWHM : Full Width Half Maximum

LSFM : Light Sheet Fluorescence Microscope

M^a : Magnification

NA : Numerical Aperture

SIM : Structured Illumination Microscope

SHG : Second Harmonic Generator

SPIM : Selective Plane Illumination Microscope

WD : Working Distance

Chapter 1

INTRODUCTION

1.1 *Optical Microscopy Essentials*

Microscopy is an examination method used to image and analyze tiny objects that are beyond the limit of what the naked eye can resolve. Optical microscopy is one of the leading type of microscopy besides electron and scanning probe microscopy. Optical microscope is the main instrument for this technique. The fundamental element for a simple optical microscope is known as lens which its origin date back to early 750 BC[19]. As an example for one of the known earliest lenses, the Nimrud lens (Figure 1.1), which was discovered by Austen Henry Layard in 1850, was assumed to be utilized such as burning-glass and magnifying glass[27].



Figure 1.1: The Nimrud lens[27]

Although the knowledge of lenses was so ancient, the rise of modern optical mi-

croscopes began in seventeenth century. The first compound microscope was invented by Zacharias Janssen by combining two lenses in a drawtube in 1595[9]. The word compound refers that more than a single lens is used to built microscope.



Figure 1.2: The first compound microscope[12]

Those kind of instruments are known as typical microscopes. Robert Hooke was the one of the most well-known scientist who built and improved a compound microscope to investigate biological sample by adding customized elements in 1665[38].

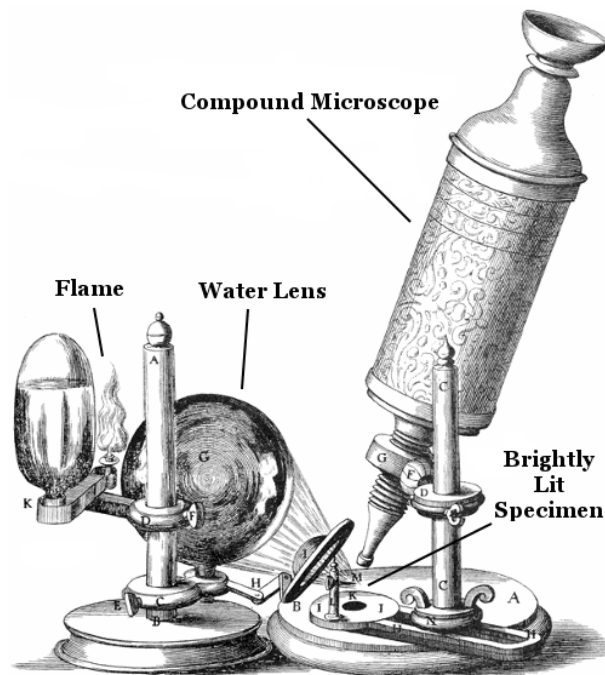


Figure 1.3: Robert Hooke's compound microscope[38]

11 years after Robert Hooke's design of compound microscopy. Antonie van

Leeuwenhoek, a Dutch textile merchant with an interest in science, is the one who observed the bacteria by making a microscope[1]. Later on, many scientist contributed to the improvement and development of optical microscopy. In addition to prior development, many different kind of microscopy techniques have been established according to the need. Bright-field microscopy[30], dark-field microscopy[30] and fluorescence microscopy are the most famous types of microscopy techniques.

1.2 Fluorescence Microscopy

As a branch of optical microscopy, fluorescence microscopy is a method to image fluorescence samples by exciting them with certain wavelengths. The historical development of fluorescence microscopy started after 1845 by Sir Frederik William Herschel who observed the fluorescence property of a quinine solution under sunlight[11].



Figure 1.4: The photograph of florescent effect of quinine solution by Malte Renz[11]

Detailed explanation for this phenomena has been given by Sir George Stokes in 1852[42]. He was the first observer of mineral flourspar. Basically, the sample emits a lower energy light after it is stimulated by absorbing a certain energy light via fluorophores. In terms of wavelength, emitted light has longer wavelength. Molecular structure of the sample determines at what certain wavelength the sample excited and emitted. The difference between excitation and emission wavelengths is known as Stokes Shift[36].

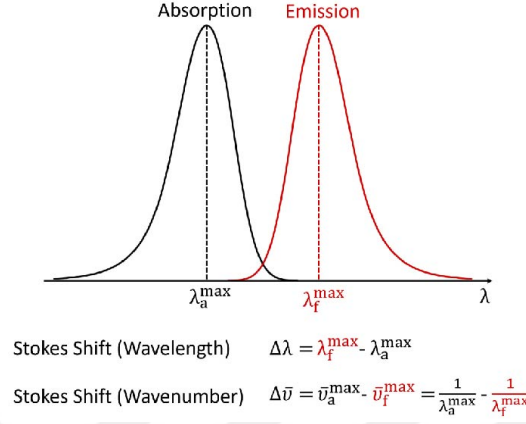


Figure 1.5: Explanation of Stokes shift[3]

The first fluorescence microscope has been introduced in the early 1900s by Carl Zeiss and Carl Reichert[42]. Traditional fluorescence microscopes can be addressed such as epifluorescence microscope, LSM, confocal microscope and two-photon excitation microscope.

1.3 Optical Sectioning

Optical sectioning is a process to generate a three dimensional image reconstruction of a sample as in mechanical sectioning. One significant advantage of optical sectioning in biomedical research as opposed to mechanical sectioning is allowing the sample to be imaged with negligible or no harm while keeping it alive.

In biomedical sciences, assortments of the optical sectioning innovations has been devised, developed and utilized in the course of recent decades[18]. The three most common microscopy techniques which is used for optical sectioning to acquire high quality images are confocal microscopy, light sheet fluorescence microscopy and two-photon microscopy[8]. Among them, light sheet fluorescence microscopy has been popular because of its virtues such as reducing image acquisition time, less photo-bleaching and improved spatial resolution[41]. An exemplary optical sectioning of a sample is given in the following figure;

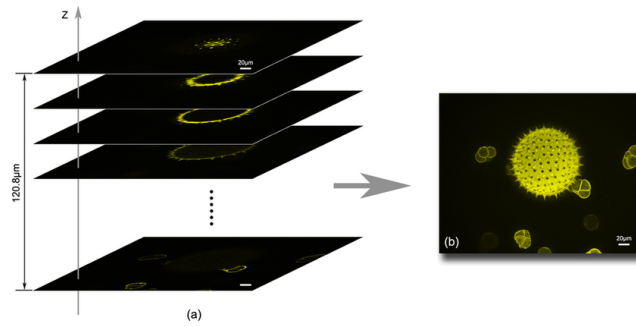


Figure 1.6: Optical sectioning of a pollen grain a) Stack of images b) Maximum intensity projection of the images[10]

In light sheet fluorescence microscopy, optical sectioning is achieved by illuminating one selected plane with a thin sheet of light. It allows for executing rapid volumetric scans of the sample. By rotating or translating the sample along the focal axis of the light sheet, images of the selected planes are collected and processed to create a three-dimensional reconstruction.

Chapter 2

LIGHT SHEET MICROSCOPY

2.1 *Fundamentals of Light Sheet Microscopy*

The first microscope that utilized the method of light sheet microscopy was introduced in 1902 by Siedentopf and Zsigmondy[39]; however, the popularity of light sheet microscopy has increased in the late 20th century. Their pioneer design for the ultra-microscope is given in the following figure (Figure 2.1). With the idea of employing sunlight as a thin-light sheet, their intention was to observe and determine the diameter of colloidal gold nanoparticles in glass[25].

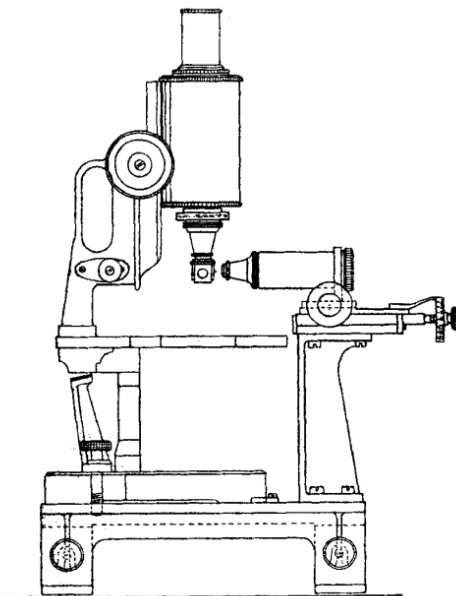


Figure 2.1: The earliest light sheet microscope developed in 1903[39]

In 1925, the Nobel Prize in Chemistry was awarded to Zsigmondy for his research on both improvement of the ultra-microscope and colloid solutions[40]. Light

sheet microscopy technique has been continued to be utilized during 20th century, particularly in material science and chemistry such as extended deep field photography[34] and deep focus microscopy[45][31]. Then, large biological samples coupled to a fluorescent tag were examined with light sheet fluorescence microscopy which was developed by Voie, Burns and Spelman in 1993[44]. Voie *et al.* established an original technique for light sheet microscopy which is known as orthogonal-plane fluorescence optical sectioning (OPFOS) to reconstruct the three-dimensional model of the guinea pig cochlea[44]. In their method, a laser beam is shaped as a thin sheet of light by employing a cylindrical lens to illuminate a narrow layer portion of the specimen and imaged with a camera[25]. The popularity of the method, improvement on its designs and work with light sheet microscopy, was accelerated after about a decade later due to studies on biological applications.

Selective plane illumination microscopy, which is a light sheet based microscopy technique, was developed by Huiskien *et al.* in 2004. SPIM technique combines two orthogonal arms that are respectively for illumination and detection to obtain optical sectioning. Due to advantages of light sheet microscopy such as low phototoxicity and high resolution at extensive penetration depths, SPIM displayed that light sheet microscopy is a beneficial method for multidimensional imaging of biological specimens[15].

2.2 Working Principle

Unlike the most types of microscopes, light sheet microscopy has two different optical paths which are respectively the detection and the illumination axes (Figure 2.2). Those axes are orthogonal to each other. The key principle of light sheet microscopy is illuminating the sample, which is located at the junction of the detection and illumination axes, with extremely thin sheet of light from one of the side of it and collecting the data by the detection axis[15].

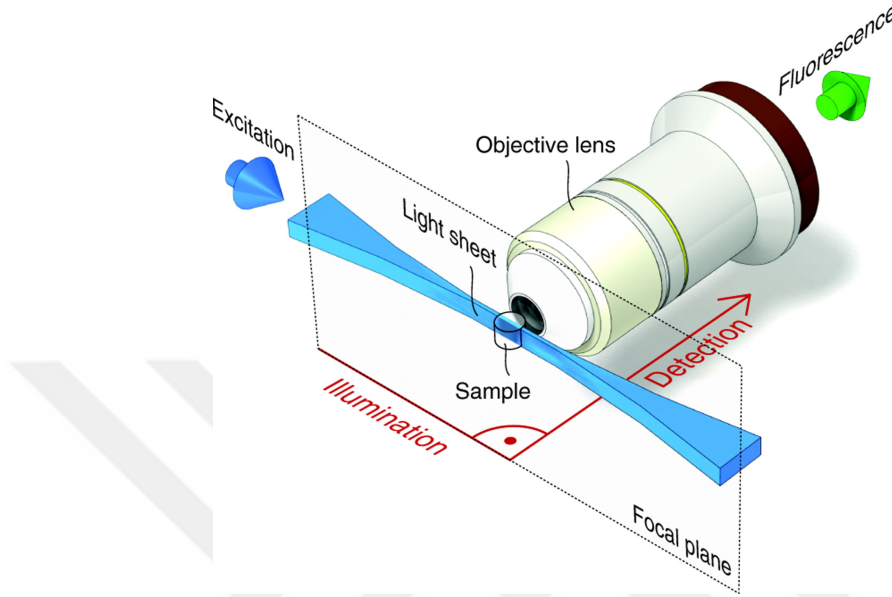


Figure 2.2: Detection and illumination paths[23]

2.2.1 Light Sheet Generation

In light sheet fluorescence microscopy technique, there are mainly 2 approaches to generate a thin sheet of light. Those approaches are known respectively as DSLM, which stands for Digital Scanned laser Light sheet fluorescence Microscopy, and light sheet generation by using cylindrical lens.

2.2.1.1 Using a Scanned Beam

The key concept of DSLM is generating a virtual light sheet by scanning a diffraction-limited laser beam by using computer assisted scan mirrors[35]. In this approach, a Gaussian beam is scanned at the illumination objective's back focal plane utilizing a mirror to generate a plane of light[2]. Generated light sheet is scanned across the sample to obtain images. The main benefit of this method is the intensity distribution of light sheet for each plane is uniform[16].

In addition to uniformity of intensity, the fact that there is no need for an aperture to form a light sheet is another advantage which increases the image quality by

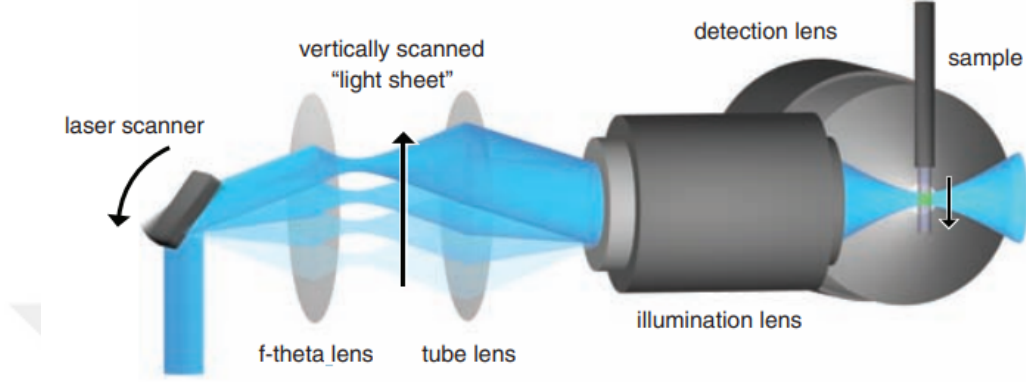


Figure 2.3: Illumination mechanism of DSLM set-up[16]

reducing optical aberrations[16]. Another benefit is the ability to modify the intensity of the illumination patterns as in SIM[33].

2.2.1.2 Using Cylindrical Lens

Instead of a dynamic virtual light sheet which can be generated by scanning a laser beam, the most clear methodology is to produce a static light sheet with a cylindrical lens. The main difference between a spherical lens and a cylindrical lens is the shape of the beam at the focus; instead of a spot, a cylindrical lens focuses light into a line[24].

Figure 2.4 displays both static and virtual light-sheet generation.

2.2.2 Illumination Mechanism

In this microscopy technique, a narrow light sheet is utilized to illuminate a particular plane of the sample. The main concept is intersecting the center of the light sheet with the focal plane of the detection optics. The selective illumination of the sample reduces the photo-bleaching effect because only one plane is exposed to light during stack image recording. The thickness of the light sheet plays several important roles such as determining the axial resolution of optical sectioning.

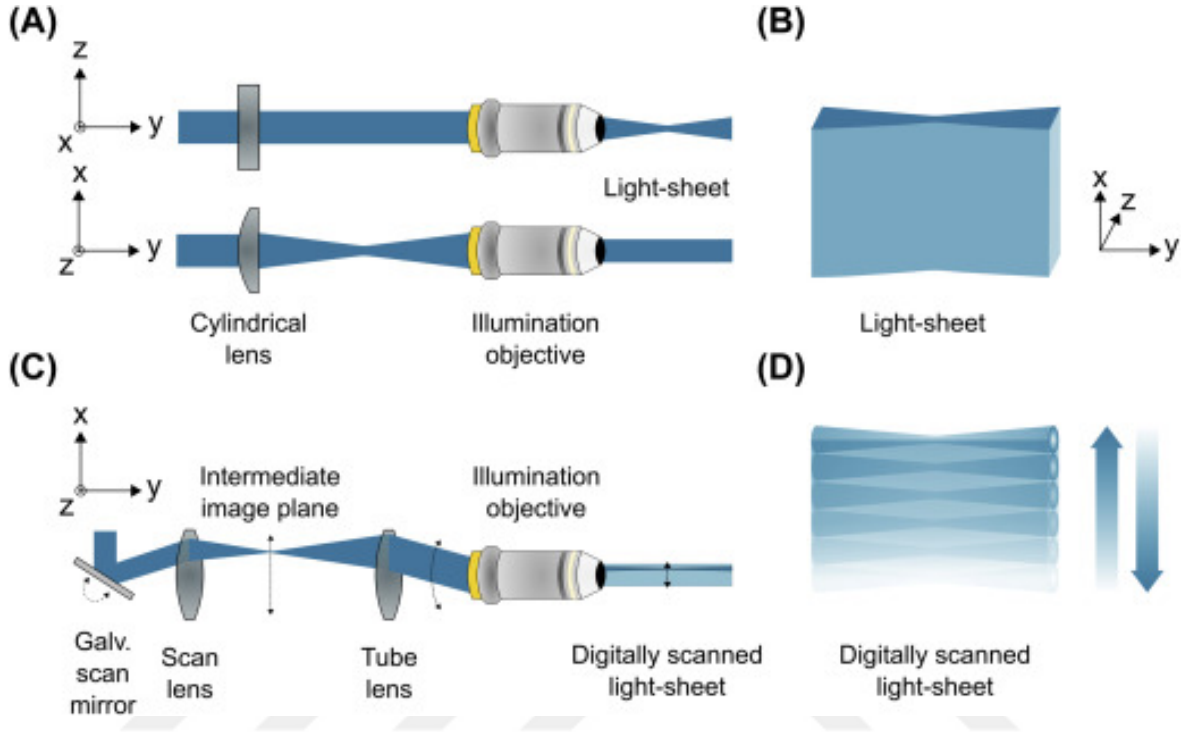


Figure 2.4: (A–B) A static light-sheet generation with cylindrical lens (C–D) A digitally scanned light-sheet generation[26]

In addition to axial resolution, there is a certain correlation between light sheet thickness and Rayleigh range.

2.2.3 Detection Mechanism

The detection optics of LSFM is usually same with the wide-field microscope which contains an infinity corrected microscope objective that collects light from the illuminated section of the sample located at the focal plane of it. Then, that light passes through an emission filter to eliminate scattered laser light from the sample. Later on, a tube lens focuses the light on the sensor of the camera. The advantage of the LSFM over confocal microscopy is the fast imaging of the sample. In confocal microscopy, the whole plane must be scanned point by point. However, as it seen in the following figure, the entire plane is imaged with a single frame.

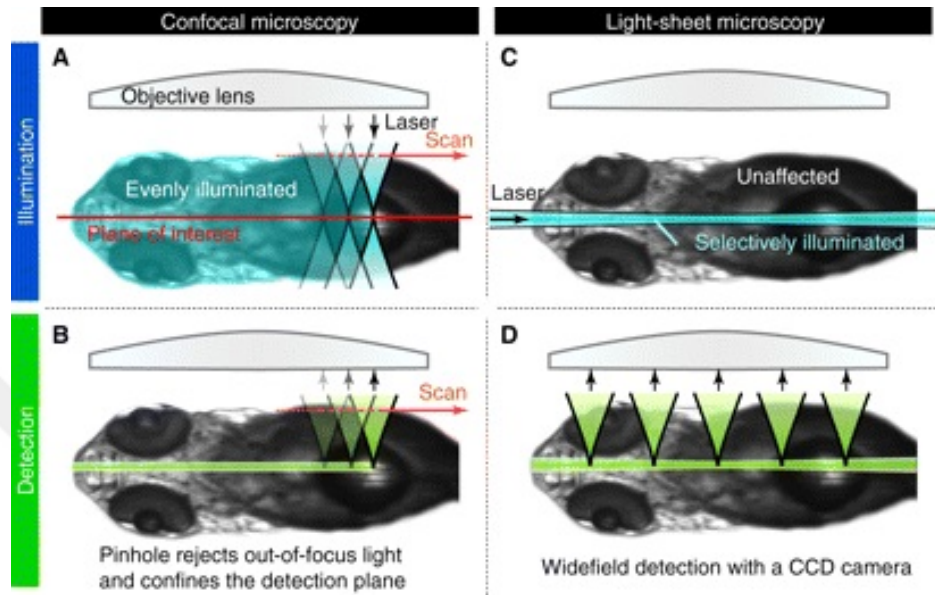


Figure 2.5: Comparison of confocal microscopy (A, B) and LSFM (C, D) in terms of illumination and detection[23]

The lateral resolution is determined with the emission wavelength of the fluorescent dye and the NA of the detection objective lens as in wide-field microscopy.

Chapter 3

EXPERIMENTAL SET-UP

3.1 *Light Sheet Formation*

In this study, the thin light sheet has been generated by using a cylindrical lens (LJ1695RM) with focal length of 50 mm. Initially, the laser beam is expanded by using a telescope which was built via two Plano-convex lenses whose focal lengths are respectively 25 mm and 50 mm in a 1 to 2 configuration. After the laser beam has been expanded by the telescope, it passes through a variable rectangular slit which allows to determine the beam size that goes across the cylindrical lens. Therefore, the numerical aperture of cylindrical lens and the dimensions of the light sheet can be adjusted via a rectangular slit. The cylindrical lens focuses the expanded laser beam to a thin vertical line onto the corner mirror which directs the light to the sample plane.

In the sample plane, the directed beam first passes through a telescope to be demagnified. This telescope halves the size of the beam. After the beam passes through the telescope and is reduced in size, it focuses on the sample via an illumination objective which could be a lens or a microscope objective.

3.1.1 *Experimental Set-Ups*

In this work, there were mainly two different configurations for LSFM. The first configuration contains two orthogonal arms which are parallel to the optical table. One arm was for detection and the other one was to illuminate the sample. In the second configuration, the sample is illuminated from both sides via separate light sheets. Moreover, the axis of detection arm has been changed to be perpendicular to the optical table by preserving its orthogonality with the illumination arms.

3.1.1.1 One-Sided Illumination

During this study, one-sided illumination for the LSM was used for a large period of time. Main structure of the experimental set-up remained the same throughout all experiments. The following figure shows the one-sided illumination for the LSM where all of the components are labeled.

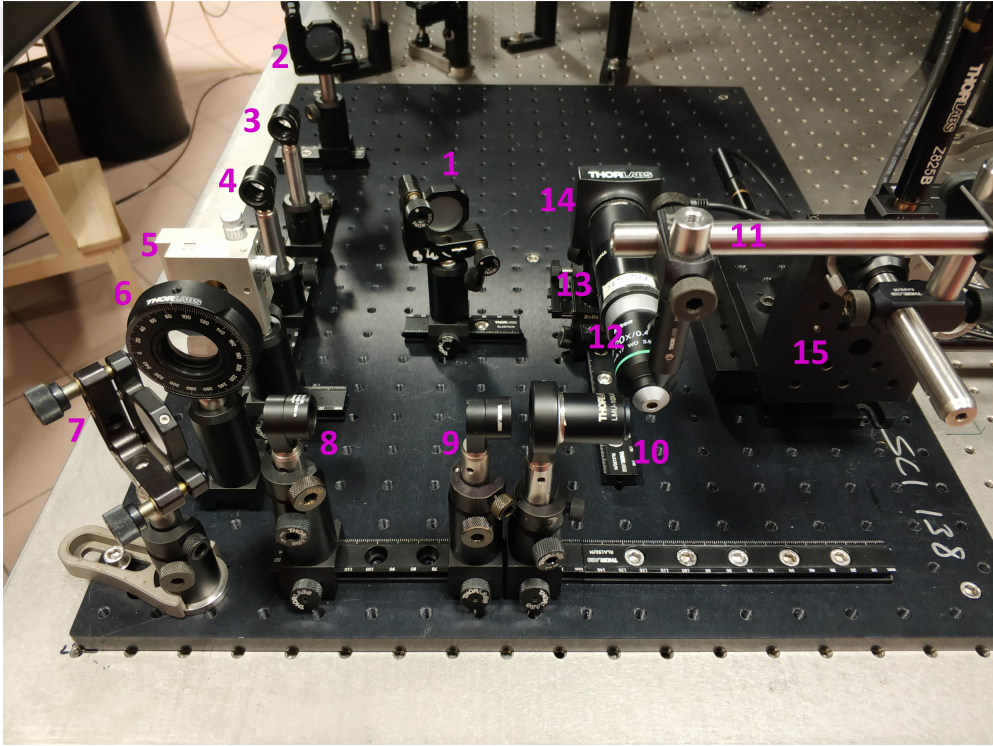


Figure 3.1: The photograph of one-sided illumination experimental set-up

Although the light sources do not appear in the Figure 3.1, both a Coherent Chameleon tunable Laser with a SHG and a diode laser were used interchangeably. Operation wavelength for laser sources was 525 nm. The explanation of the other components are given in the following;

1. Mirror No.1 (BB1-E02): To direct and align the light source
2. Mirror No.2 (BB1-E02): To direct and align the light source
3. Lens No.1 (AC127-025-A-ML): To expand the laser beam
4. Lens No.2 (AC127-050-A-ML): To expand the laser beam

5. Rectangular Slit: To adjust the Rayleigh range and sheet thickness
6. Cylindrical Lens (LJ1695RM): To generate the light sheet.
7. Mirror No.3 (BB1-E02): To direct the light source to the illumination axis
8. Lens No.3 (AC127-050-A-ML): To resize the laser beam
9. Lens No.4 (AC127-025-A-ML): To resize the laser beam
10. Objective (LMU-10X-UVB): To focus the light sheet
11. Sample Holder: Customized optomechanical design for holding the sample
12. Objective (CFI Achromat LWD 20X): To collect the fluorescence beam from the sample
13. Achromatic Doublet (AC254-050-A-ML): To focus the fluorescence beam to the camera sensor
14. CMOS Camera (DCC1545M): To obtain images from the sample
15. Motorized Stage (MT1-Z8): To adjust the position of the sample

The schematic drawing of the one-sided light sheet illumination is given in the following figure.

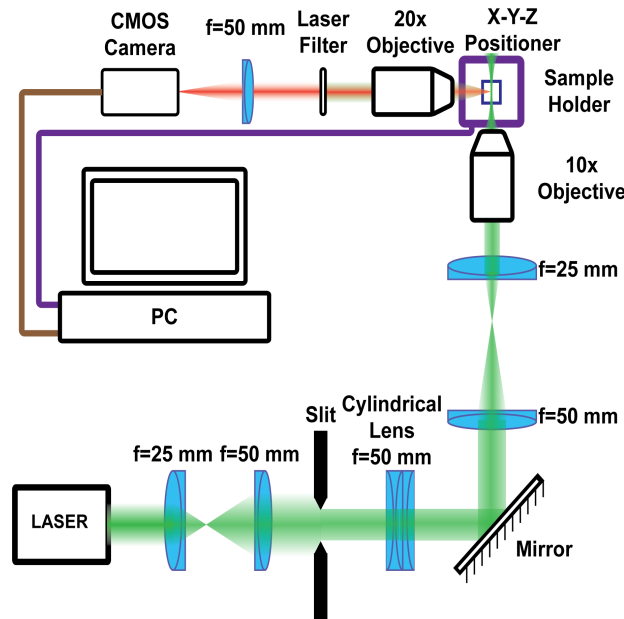


Figure 3.2: Schematic diagram of the one-sided illumination for the light sheet fluorescent microscope

Several different camera sensors have been used during experiments. In the section 3.6, detailed information about cameras is given by comparing image quality with respect to each of them.

3.1.1.2 Two-Sided Illumination

In order to obtain better imaging results from thick samples, two-sided illumination is crucial. Because of scattering, light sheet could not penetrate into the far side of the sample. This situation gives rise to low signal-to-noise ratio on that side of the image. Therefore, in the second part of the experiments, optical set-up for two-sided illumination of light sheet microscopy was built. In addition to one-sided illumination experimental set-up, a beam splitter was added to obtain a second light sheet from the same source. The photograph and brief explanation of the components are given in the following figure.

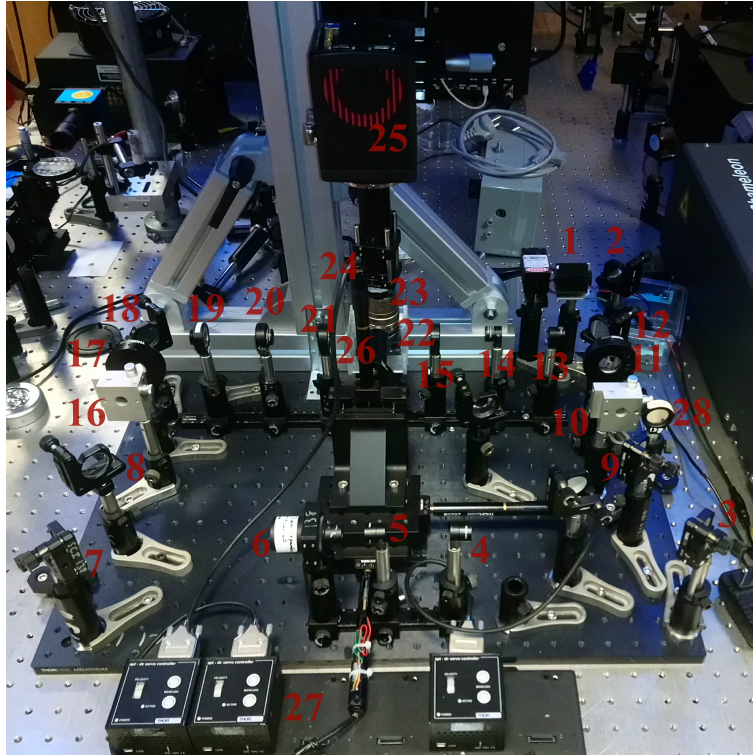


Figure 3.3: The photograph of two-sided light sheet illumination experimental set-up

1. Diode Laser (525 nm): To be a light source
2. Mirror No.1 (BB1-E02): To direct and align the laser light
3. Mirror No.2 (BB1-E02): To direct and align the laser light
4. Lens No.1 (AC127-025-A-ML): To expand the laser beam
5. Lens No.2 (AC127-050-A-ML): To expand the laser beam
6. Beam Expander: To expand the laser beam
7. Mirror No.3 (BB1-E02): To direct and align the laser light
8. Optical Beam Splitter (BSW10): To split the beam into two
9. Mirror No.4 (BB1-E02): To direct and align the laser light
10. Rectangular Slit: To adjust the Rayleigh range and the light sheet thickness
11. Cylindrical Lens (LJ1695RM): To generate the light sheet.
12. Mirror No.4 (BB1-E02): To direct the light source to illumination axis
13. Lens No.3 (AC127-050-A-ML): To expand the laser beam
14. Lens No.4 (AC127-025-A-ML): To expand the laser beam
15. Best Form Lens (LBF254-040-A): To focus the light sheet
16. Rectangular Slit: To adjust the Rayleigh range and the light sheet thickness
17. Cylindrical Lens (LJ1695RM): To generate the light sheet.
18. Mirror No.5 (BB1-E02): To direct the light source to illumination axis
19. Lens No.5 (AC127-050-A-ML): To expand the laser beam
20. Lens No.6 (AC127-025-A-ML): To expand the laser beam
21. Best Form Lens (LBF254-040-A): To focus the light sheet
22. Objective (CFI Plan Apochromat 10XC Glyc): To collect fluorescence beam from the sample
23. Achromatic Doublet (AC254-150-A): To focus the image on the camera
24. Emission Filter (FEL0550): To filter the laser light contribution in the image
25. Camera (ORCA Flash 4 v2.0): To obtain the images from the sample
26. Motorized Stage (MT1-Z8): To adjust the position of the sample
27. Servo Motor Controller (KDC101): To control motorized stage
28. Excitation Filter (AC127-025-A-ML): To filter laser source

In this configuration, the light sheet has been formed by cylindrical lenses as in the one-sided illumination one. Also, another beam expander has been employed to resize the source beam enough to fill the back aperture of the cylindrical lenses. There is a correlation between the area filled in the back aperture of the cylindrical lens and the thickness of the light sheet. The larger area in the back aperture filled, the thinner the light sheet. After the second beam expander (1:2.5), the beam is directed to the beam splitter via a corner mirror. The beam splitter divides the incoming beam into two beams so that the intensities are the same and direct them to the illumination axes in the set-up. The schematic drawing of the two-sided light sheet illumination is given in the following figure.

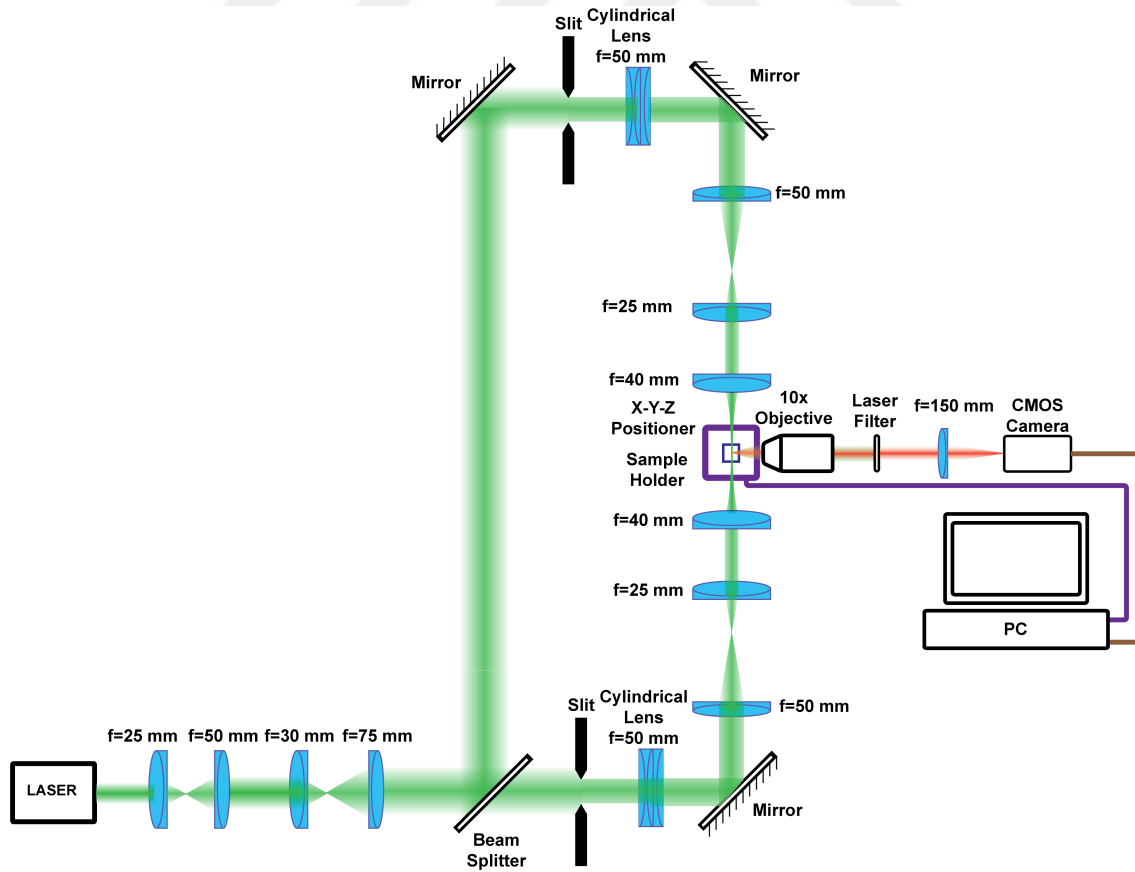


Figure 3.4: Schematic diagram of the two-sided illumination for the LSM

In all the experiments in which two-sided light sheet illumination experimental

set-up was used, the samples were immersed in glycerol. Thus, a custom sample chamber was designed and further details in the section 3.3.

3.1.2 Sheet Thickness Measurement

In this microscope, the thickness of the light sheet has been measured by recording images of the light sheet by translating a CMOS sensor (Pointgrey Camera) along the light sheet axis with incremental steps (Figure 3.1) for both one-sided and two-sided illumination. In order to prevent saturation of the camera pixels, a variable natural density filter has been used. During this measurements, the rectangular slit was wide open to obtain thinnest light sheet at joint focal plane of the illumination and detection optical components. In addition, the light sheet was focused by a best form lens (LBF254-040-A).

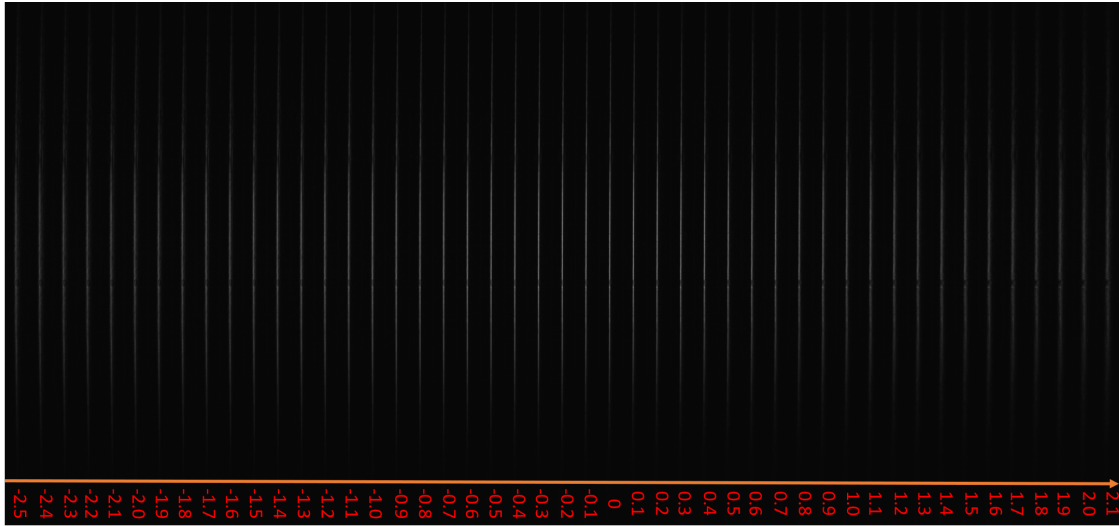


Figure 3.5: Light sheet images along the sheet axis for one-sided illumination. Axis indicates distance between light sheets in millimeters

In order to obtain the light sheet thickness value from these images, an intensity profile has been generated in one axis by using a MATLAB code (Appendix A) at each step. FWHM of the intensity profile characterizes the thickness of the thickness of the light sheet. The pixel size of the sensor is $5.8 \mu\text{m} \times 5.8 \mu\text{m}$. Analysis of the

images taken with the one-sided illumination configuration (Figure 3.2) implies that the thickness of light sheet is about $14.6 \mu\text{m}$.

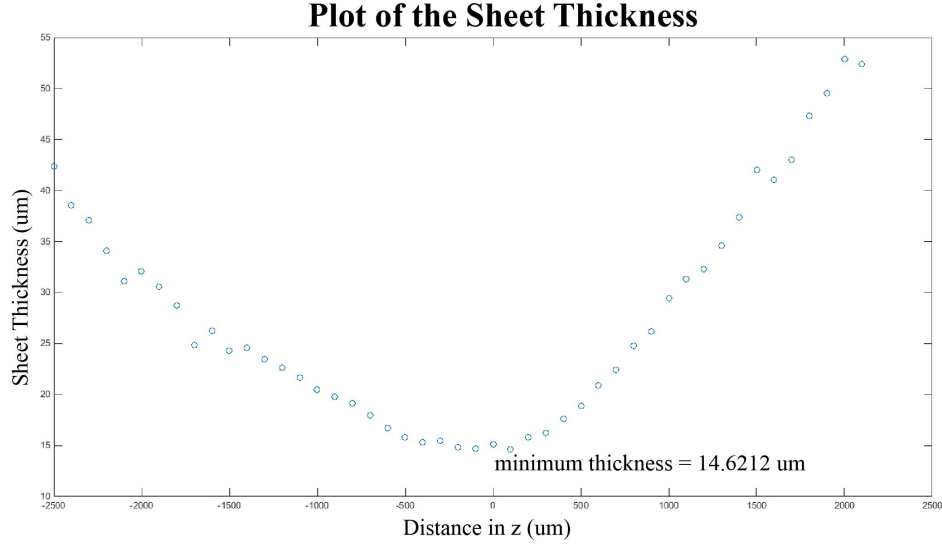


Figure 3.6: The light sheet thickness measurement result

3.1.3 Zemax Simulations

There were several Zemax simulations performed for both light sheet thickness and optical item selection. There were two different elements to focus the light sheet into the sample. Those elements are respectively a 10x microscope objective (LMU-10X-UVB) and a best form lens (LBF254-040-A). Due to the physical limitations, the light sheet thickness which was focused via the microscope objective could not be directly measured by using a camera. Therefore, a PDMS sample which was prepared with fluorescent dye was used to determine the light sheet thickness, and a Zemax analysis was performed to justify this measurement.

Prepared PDMS sample was imaged (Figure 3.7) with a Nikon microscope objective (CFI Achro LWD 20x). The line intensity profile was obtained by scanning the sample in $2\mu\text{m}$ steps.

The FWHM of the line intensity profile was calculated to find the light sheet

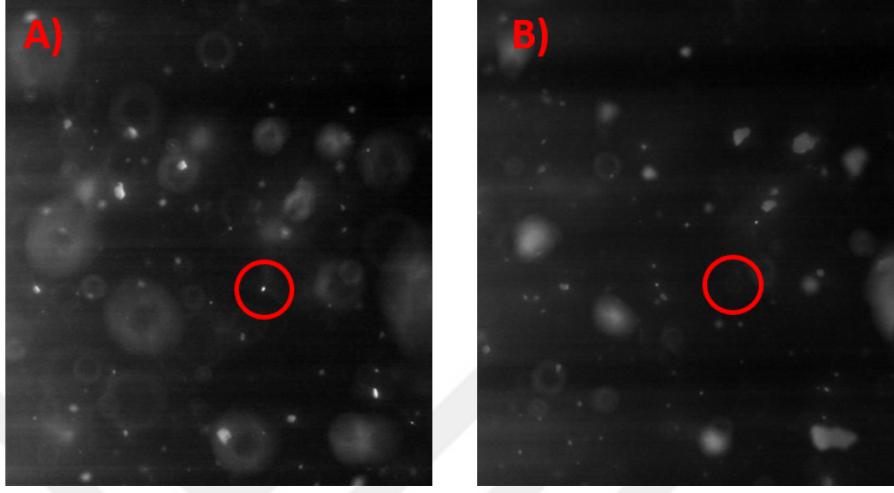


Figure 3.7: Stained PDMS sample. Layer separation is $20\ \mu\text{m}$

thickness (Figure 3.8) by choosing one dye particle which was marked with a red circle.

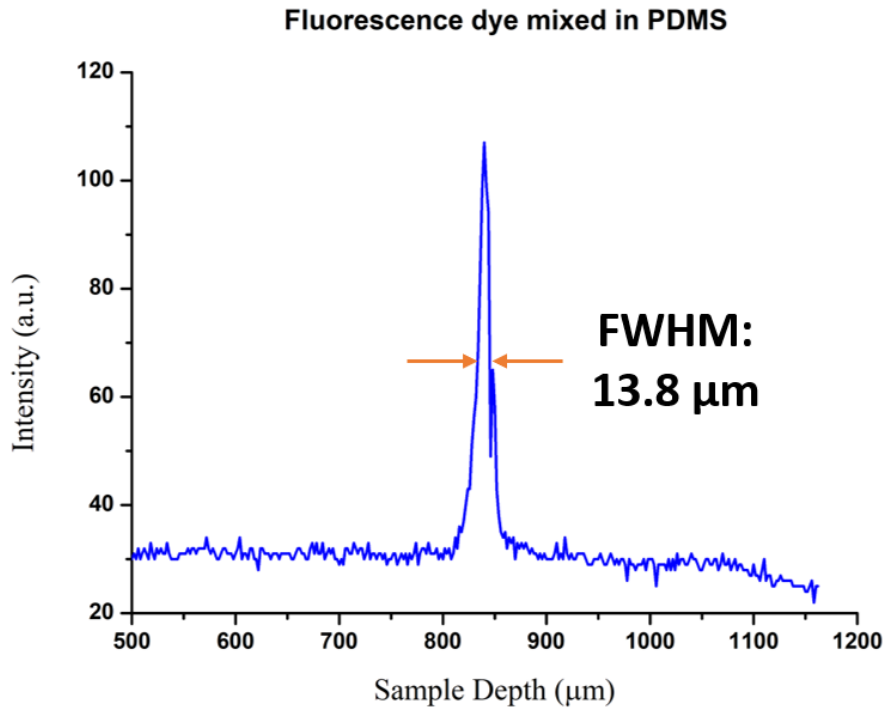


Figure 3.8: The line intensity profile of the PDMS sample

According to this measurement, the light sheet thickness was found to be about $13.8\mu\text{m}$ at the focus of the lens. The results of the simulation which was performed by Zemax can be found in the following figure (Figure 3.9).

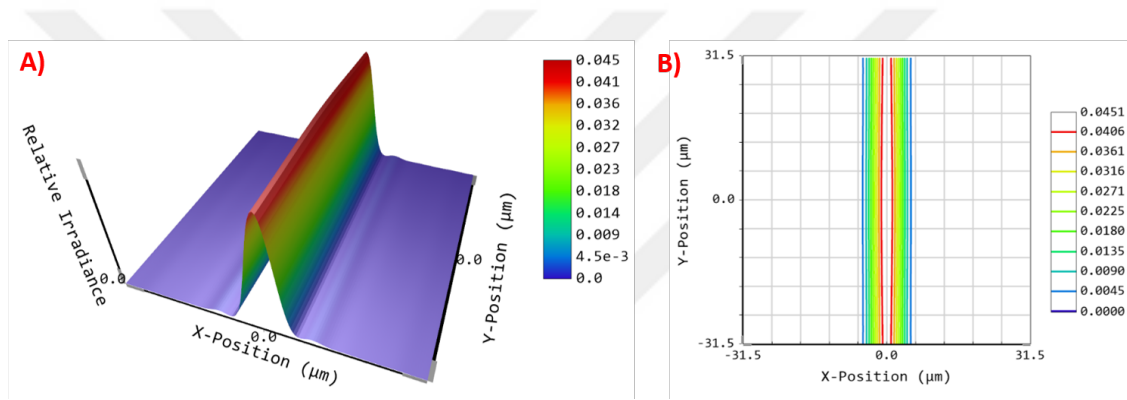


Figure 3.9: 3D beam intensity profile at focus A) Three-dimensional B) Two-dimensional

According to Zemax analysis, the light sheet thickness is supposed to be around $8\mu\text{m}$. Some reasons to explain the difference between the actual value and the simulation value can be as follows; precision in alignment and defects of manufactured optical elements.

A second Zemax analysis was performed for choosing a lens to focus the light sheet. The cost of the LSFM was aimed to be low. In order to choose the lens which has the best price-performance ratio, data was obtained from the Zemax analysis given in the figure (3.10).

Suitable lenses are listed in the table (Table 3.1) below.

Item Number	Item Name	Focal Lenght (mm)	Price (€)
1	LA1560[37]	25	27,94
2	LBF254-040[4]	40	50,38
3	AL1225M[32]	25	194,82
4	AL1225G[14]	40	220,02

Table 3.1: Specifications of the suitable lenses

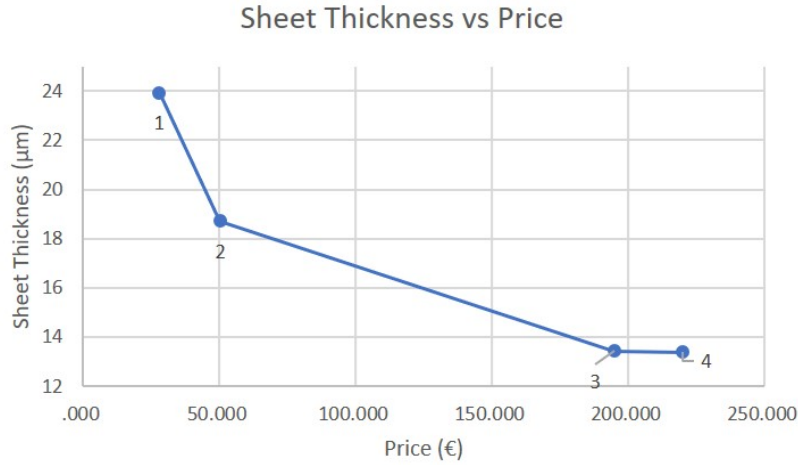


Figure 3.10: Zemax analysis to select the lens

According to data, the best form lens (LBF254-040) was selected. Estimated light sheet thickness is about 18 μm in glycerol medium.

A fluorescence bead sample (Figure 3.11) has been prepared to measure the thickness of the light-sheet for two-sided illumination experimental set-up in glycerol medium. The sample was illuminated individually from both arms to make comparisons and fine alignments. Then, the FWHM of the line intensity profile was calculated to find the light sheet thickness (Figure 3.12) by choosing one fluorescent bead which was marked with a red circle.

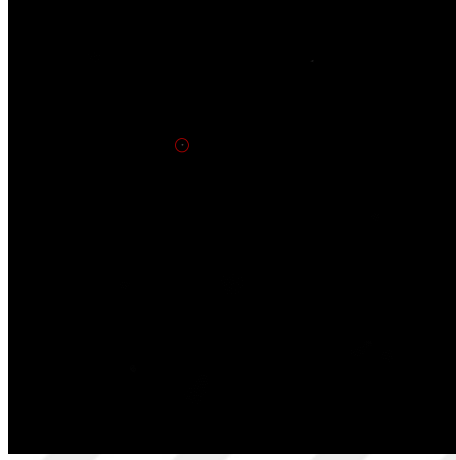


Figure 3.11: Fluorescent Bead

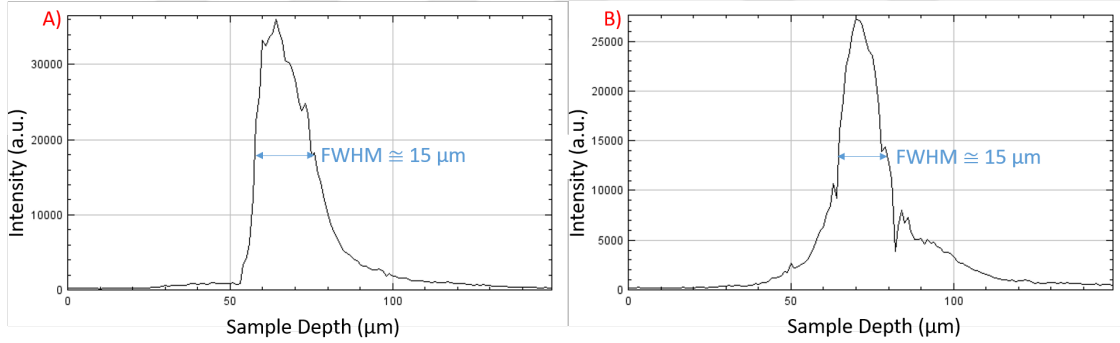


Figure 3.12: A)Illuminated from the left arm B)Illuminated from the right arm

As a result of this measurement light-sheet thickness was measured as 15 μm .

3.1.4 Microscopy Calculations

In order to check the limitations in terms of resolution for both axial and lateral directions. Firstly, the lateral resolution is given by the Rayleigh criteria. The equation for calculating the lateral resolution of the detection arm[17];

$$R_{lat} = 0.61 \frac{\lambda_{em}}{NA_{det}} \quad (3.1)$$

where;

R_{lat} is the lateral resolution,

λ_{em} is the emission wavelength of the fluorescent sample,

NA_{det} is the numerical aperture of the objective,

In the light-sheet experimental set-up;

λ_{em} : 565 nm

NA_{det} : 0.50

As a result of this calculation, the lateral resolution in the set-up is found as 0.69 μm . In this set-up the image pixel size is 0.85 μm . That means, the system is not exceeding limit of the camera sensor.

In addition to lateral resolution, axial resolution[17] can be found as

$$R_{ax} = 1.78 \frac{\lambda_{em} \cdot n}{NA_{det}^2} \quad (3.2)$$

where;

R_{ax} is the lateral resolution,

n is the refractive index of the medium,

λ_{em} is the emission wavelength of the fluorescent sample,

NA_{det} is the numerical aperture of the objective,

As a result of this calculation, the axial resolution in the set-up is found as 5.87 μm . The thickness of light-sheet is much grater than the axial resolution. Thus, the axial resolution of the experimental set-up is limited with the light-sheet.

3.2 Sample Preparation

In this study, one particular aim was to image biological samples. For this purpose, liver tissue samples, that are cleared via passive CLARITY procedure by adjusting

incubation times and polymer concentrations[20], were used. Clearing process were performed in clearing solution which includes boric acid (200 mM, pH 8,5) and 4-8% (weight/volume) sodium dodecyl sulphate (SDS) for 6-8 weeks at 37°C.

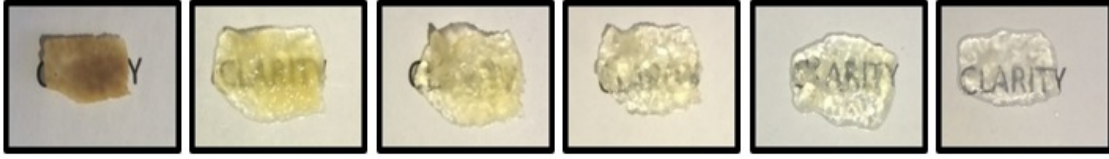


Figure 3.13: Weekly progression of liver tissue after CLARITY process

After this process, transparent tissues were stained with antibodies that target Elastin, Collagen I and other extracellular matrix proteins and appropriate secondary antibodies to obtain images by using fluorescence microscopy.

3.3 Sample Chamber

In fluorescence microscopy, one of the noise sources is the imaging medium where the sample is located. In order to minimize the noise due to refractive index mismatches with the medium and the sample, there should be a chamber which is filled with an immersion medium matching the refractive index of the sample. The refractive index of the cleared tissue samples is 1.46 which matches with the glycerol solution 86-89%.

For this study, the dimensions of the designed chamber is 7.5 cm x 5.5 cm x 3 cm rectangular box which is filled with glycerol solution (Figure 3.14). It stands on a mounting base (BA2) which is connected to a rail carrier via a post and post holder.

The blueprint of the sample chamber was designed in AutoCAD 2019 (Figure 3.15). The sample chamber was formed by laser cutting from Plexiglas material. All edges of the chamber were merged by using chloroform.

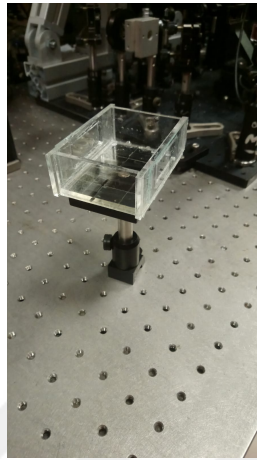


Figure 3.14: Sample chamber

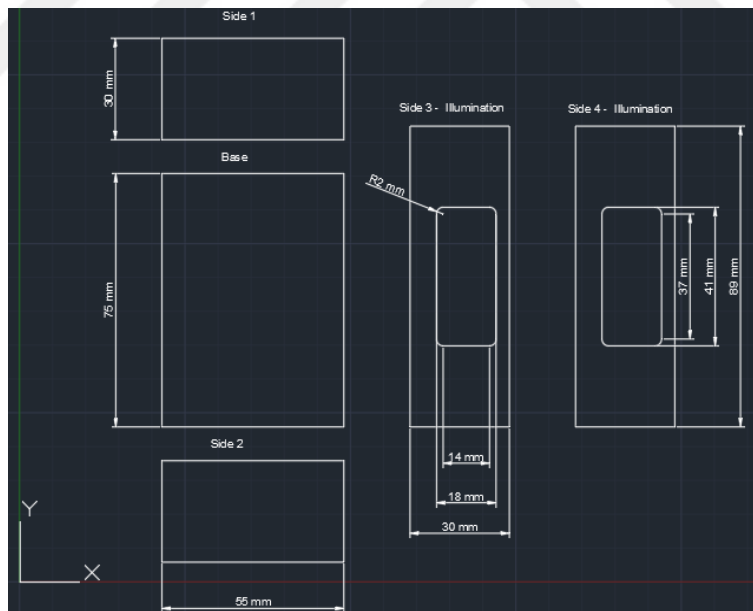


Figure 3.15: Schematic drawing of the sample chamber

3.4 Sample Holder

The most challenging part in this study is to fix the sample into the chamber. The orientation of the sample on the holder has crucial role for collecting data. One of the easiest way of holding the sample is hanging it in the focal plane of the illuminating objective.

In the beginning of this project, a customized hanging system was designed by using optical posts, right angle clamps (RA90) and compact flexure plate clamp (PC2/M). Tissue sample was pinned on a glass slide via medical rubber bands. Glass slide with the tissue was hung via a plate clamp.



Figure 3.16: The stock photograph of plate clamp[7]

This design (Figure 3.17) was a good start, but the medical rubber bands used were not very suitable for several reasons. First of all, it was incompatible because it tore the narrow tissues from where they were attached. In addition, it deformed the bottom of the tissue by compressing it on the glass. Another reason was that the attached parts of tissue could not be imaged.

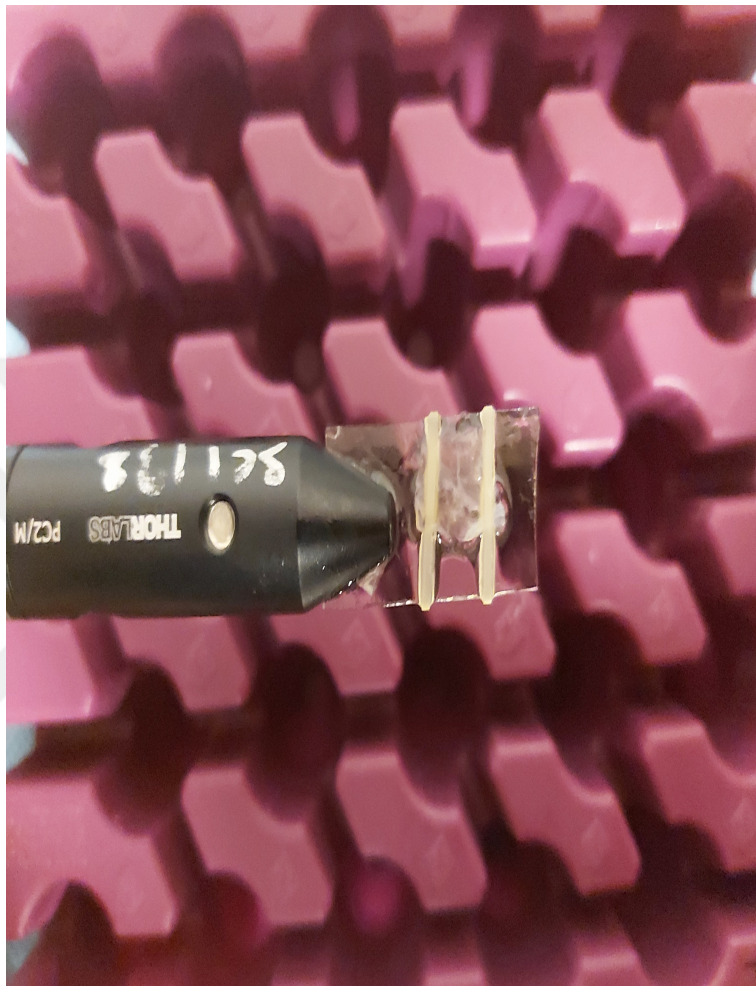


Figure 3.17: The first sample holder design

This design works effectively when the plane of the light sheet is perpendicular to the optical table. Another holder is designed for the system in which the plane of the light sheet is parallel to the optical table. One of the advantages of this system is that the tissue sample can be placed in the holder more easily because gravity is not a problem. As in the sample chamber, this holder was designed with AutoCAD 2019 (Figure 3.12) software and Plexiglas was used in its manufacturing.

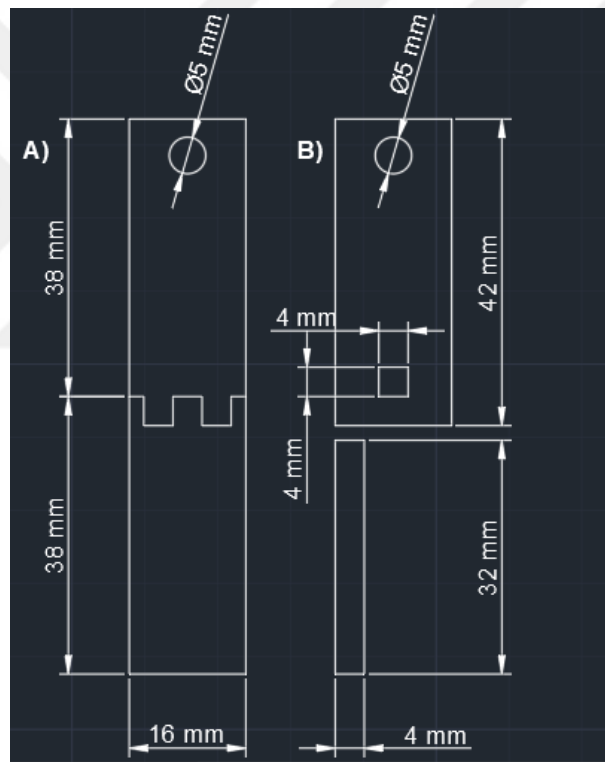


Figure 3.18: Schematic drawing of sample chamber A) For large tissues B) For narrow tissues

Due to the L-shape of this holder, tissue sample is just needed to be laid and fixed on the holder. Kapton tape is used to attached the sample on the holder. One of the most important reasons for using Kapton tape is that it does not leave any residue on the tissue and it is easy to apply. Following figure (Figure 3.19) shows a prepared holder and a tissue sample attached with tape.

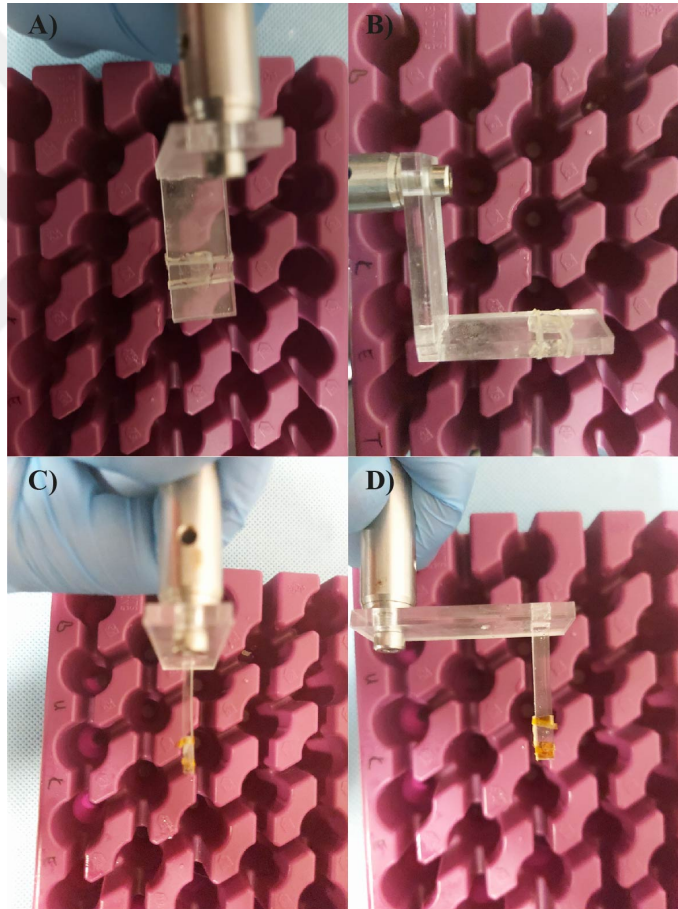


Figure 3.19: Tissue samples with holder

In addition to these two holders, a third and the last one used in the current system is designed in Fusion 360. Designed holder (Figure 3.14) was 3D printed by using an Ultimaker 2+. As the refractive indices of borosilicate glass and immersion medium are matching with each other, the tissue is being imaged by fixing into the pasteur pipette with a refractive index compatible gel. This gel contains 1% agarose and 99%

refractive index matched solution. RIMS includes nycodenz and 0.02M phosphate buffer and has pH 7.5.

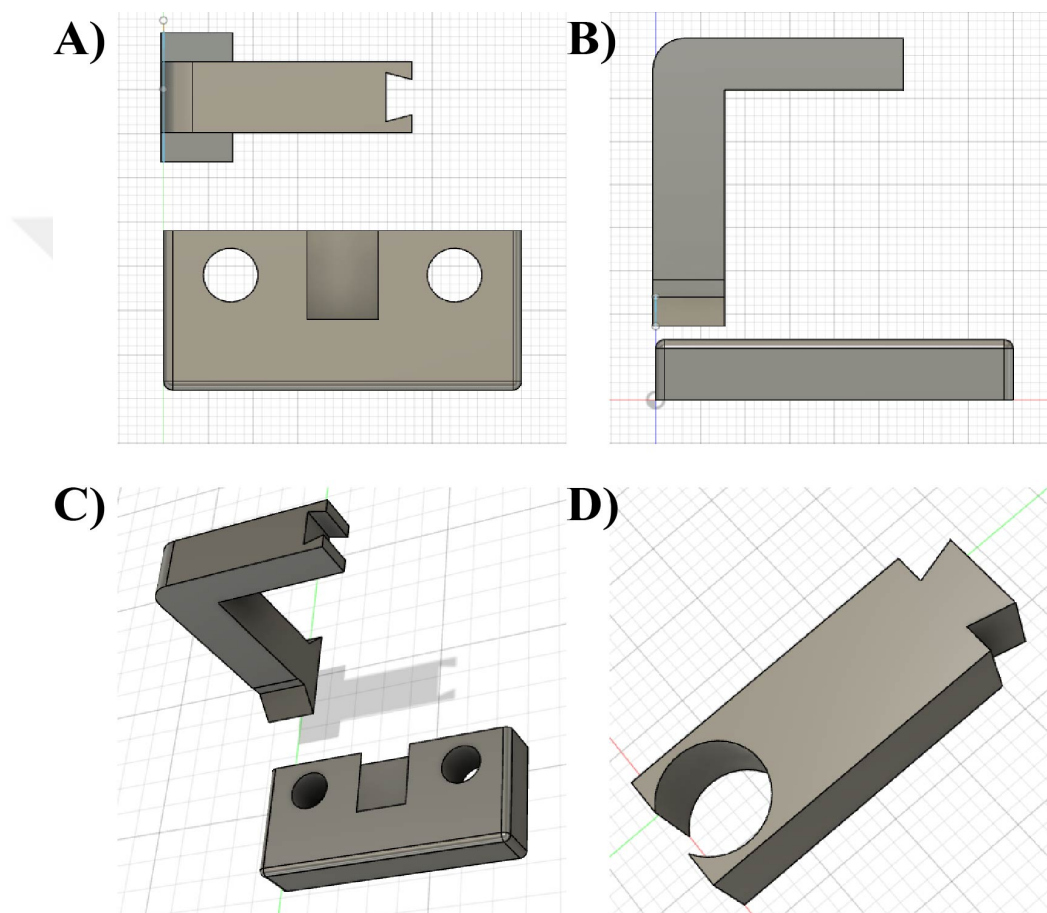


Figure 3.20: 3D drawing of the holder A) Top view B) Side view C) Isometric view D) Piece for attaching the tube

As it seen in the following figure (Figure 3.21), both ends of the tube were sealed with paraffin so that the glycerol and the gel could not contact each other. The problem with contact is that the glycerol dissolves the gel.

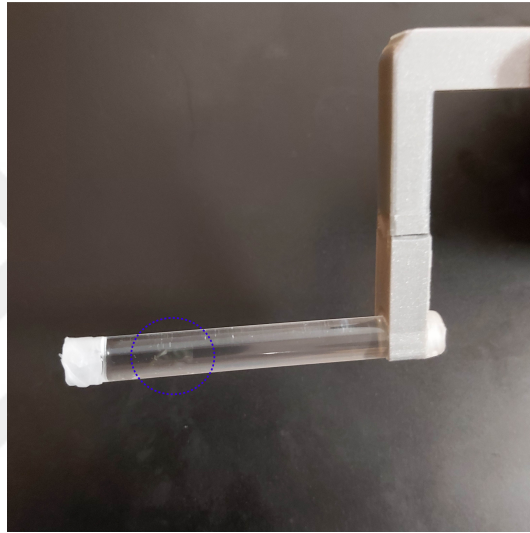


Figure 3.21: Fixed tissue in the tube

Dashed section contains the tissue. Due to refractive index matches, it is hard to detect tissue by looking through.

3.5 Image Acquisition Software

The software for image acquisition has been developed in C#. The program has been used mainly for recording images and capturing live images. The software allows the user to record a stack image in z-direction and tile images in both x and y directions within the range of the motorized stage which is 25 mm. The interface of the control program is shown in following figure.

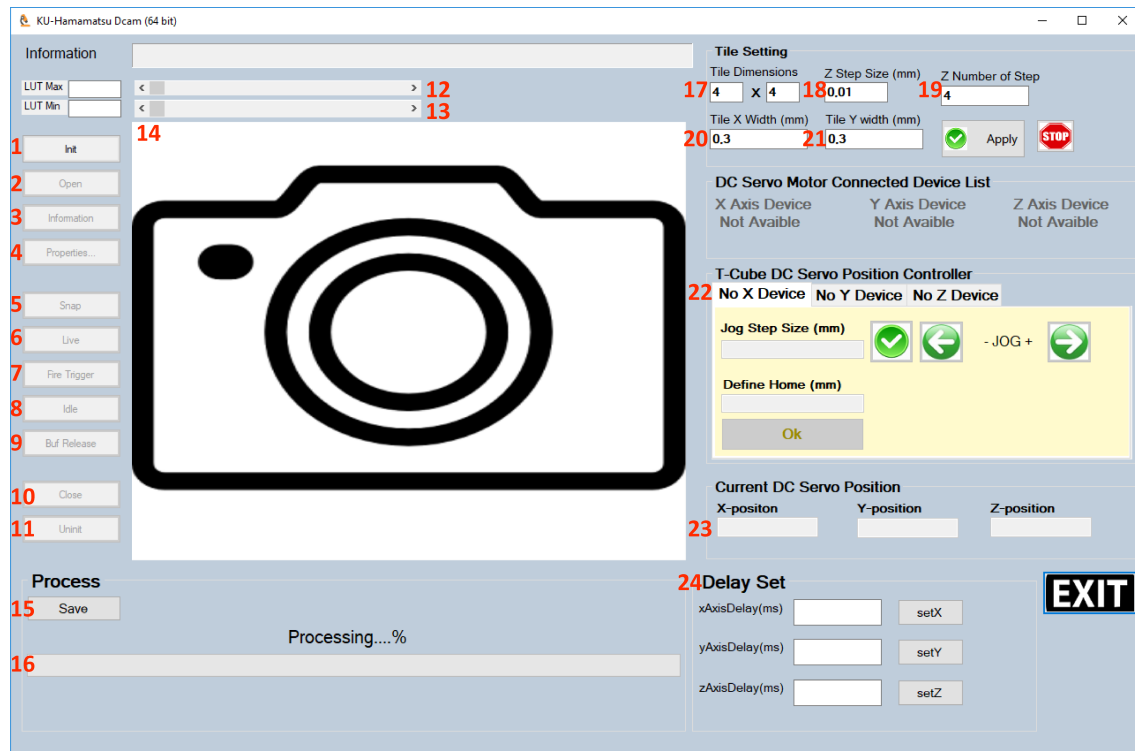


Figure 3.22: The user interface of the software

The explanation of each interactive sections in the user interface of the control program is given below;

1. Init: To initialize the camera
2. Open: To activate the camera
3. Information: To obtain data about the camera
4. Properties: To set the properties of the camera
5. Snap: To capture a snapshot
6. Live: To see the image in real time live mode
7. Fire Trigger: To use trigger
8. Idle: To standby the camera
9. Buf Release: To reset the buffer memory of the camera
10. Close: To close the camera
11. Uninit: To deselect the camera

12. LUT Max: To set camera lookup table maximum intensity value
13. LUT Min: To set camera lookup table minimum intensity value
14. Image Box: To show the camera images
15. Save: To set the save directory
16. Progression: To show progression percentage
17. Tile Dimensions: To set tile dimension in both x and y direction
18. Z Step Size: To set step size in z direction
19. Z Number of Step: To set z-stack dimension
20. Tile X Width: To set the tile width in x direction
21. Tile Y Width: To set the tile width in y direction
22. T-Cube DC Servo Position Controller: To obtain and set the data of motorized stage
23. Current DC Servo Position: To show the current position of the three dimensional motorized stage
24. Delay Set: To set the sleep time after each motorized stage movement for all directions.

3.6 Image Processing

In this study, all images were processed with Fiji ImageJ (Figure 3.21) which is an open source software for processing images.

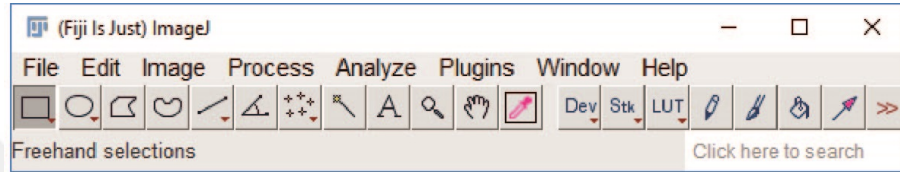


Figure 3.23: The graphical user interface of Fiji

The z-stack of tissue images were analyzed by using this software. Those analyses were getting line intensity profile and reconstruction of three-dimensional image of the tissue. There are several plugins for different purposes such as stitching. After taking tile images, the stitching plugin is used to combine them.

Chapter 4

RESULTS

As mentioned in the previous sections, several different experimental set-up configurations and optical equipment were used. In this section, obtained experimental results are presented in chronological order. For each result, different key variation are indicated. Changes in both detection and illumination optics are mainly comprise by those variations. The sample holder designed with plate clamp was used in all the results given below until it was indicated to be changed.

Changes in the experimental set-up was driven by making gradual improvements on the image quality. Initially, the preliminary results assessed the necessity of index matching for better optical sectioning. Thorlabs CMOS camera (DCC1545M) (Figure 4.1) and Thorlabs MicroSpot focusing objective (LMU-10X-UVB) (Figure 4.2) were used as critical optical components. In addition to those equipment, a 1:1 matched pair was used as detecting optics.



Figure 4.1: The stock photograph of DCC1545M[13]

The specifications of the camera are given in the following table.

Item Number	DCC1545M
Sensor Type	CMOS
Optical Sensor Format	1/2" (5:4)
Pixel Size	5.2 μ m x 5.2 μ m
Shutter Type	ERS
Frame Rate	30 fps

Table 4.1: Specifications of DCC1545M Camera[43]



Figure 4.2: The stock photograph of the LMU-10X-UVB[28]

The specifications of the illuminating objective given in following table.

Item Number	LMU-10X-UVB
AR Coating Range	240 - 360 nm
M ^a	10X
WD	15 mm
EFL	20 mm
NA	0.25

Table 4.2: Specifications of the illumination objective[29]

There were several measurement with these apparatus. Since all the results obtained were similar, one of them is given in the figure below (Figure 4.3).

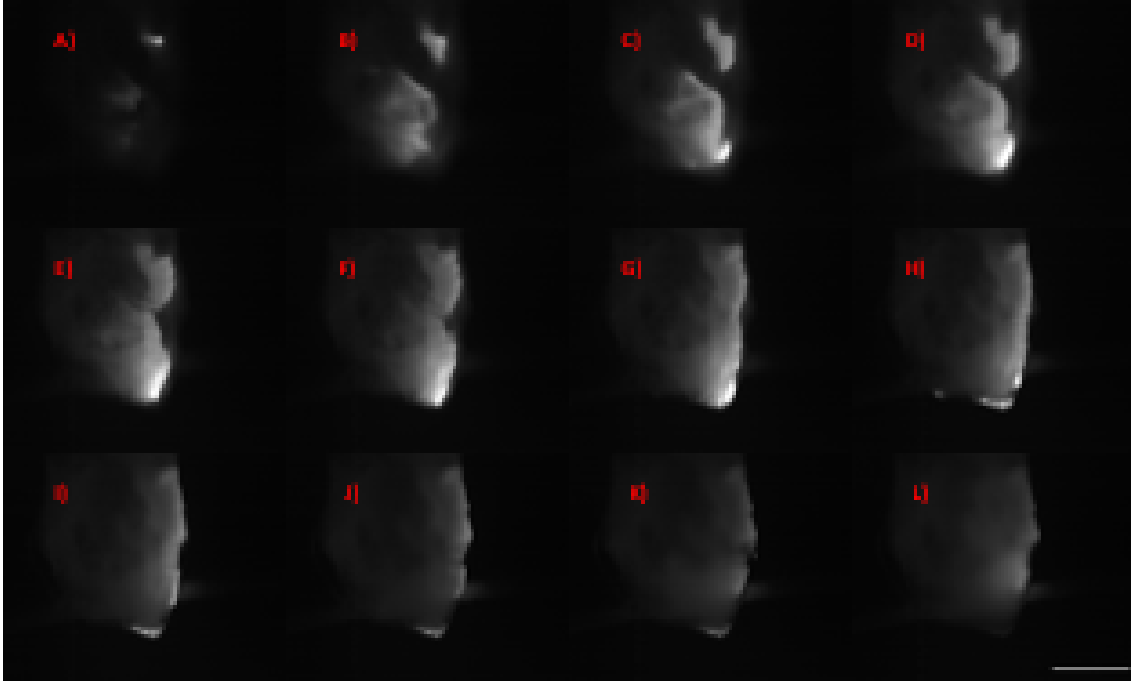


Figure 4.3: First result obtained from set-up. Distance between each image is $100 \mu\text{m}$ in z-direction. Scalebar indicates 0.5 cm

Images have been recorded by the software known as uc480 Viewer. As it is easily seen in the Figure 4.3, images are quite blurry. A few conditions that may cause this situation can be expressed as follows; the refractive indices of the medium and the sample are not compatible with each other, and poor resolution due to 1:1 matched pair. As a quantitative analysis to justify the quality of the result, following figure indicates the line intensity profiles for each image.

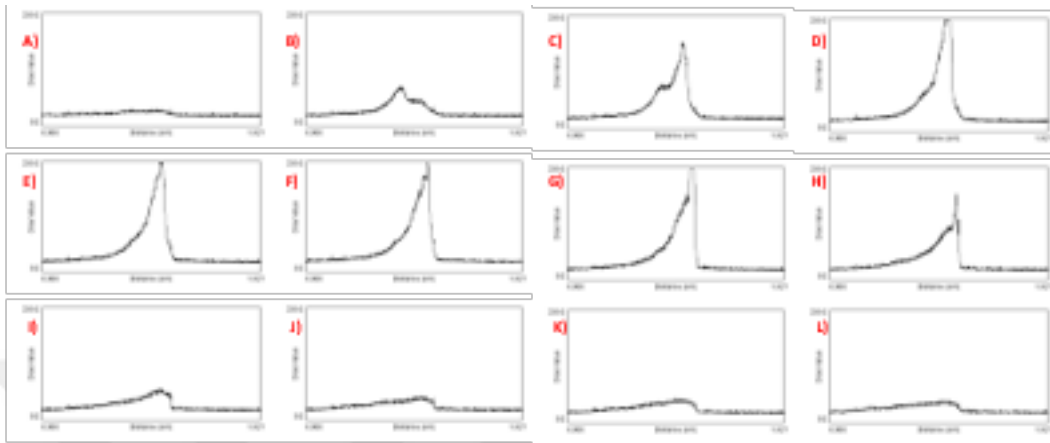


Figure 4.4: The line intensity profile of the first measurement

A good resolution estimate could be determined by analyzing the line profile. The sharper the peaks, the better the image. Based on the first result, it was decided to use a microscope objective instead of a matched pair in the detection optics. Following experiment was conducted with Nikon CFI Achro LWD 20x (Figure 4.4).



Figure 4.5: The stock photograph of the Nikon CFI Achro LWD 20x[5]

The specifications of this microscope objective are given in the Table 4.3.

Item Number	CFI Achro LWD 20x
Type	Achromat
M ^a	20X
WD	3.90 mm
Type	Infinity Corrected
NA	0.40

Table 4.3: Specifications of Nikon CFI Achro LWD 20x[5]

One of the experimental results which was taken with this objective is given in the Figure 4.6. The imaging medium was air for this measurement.

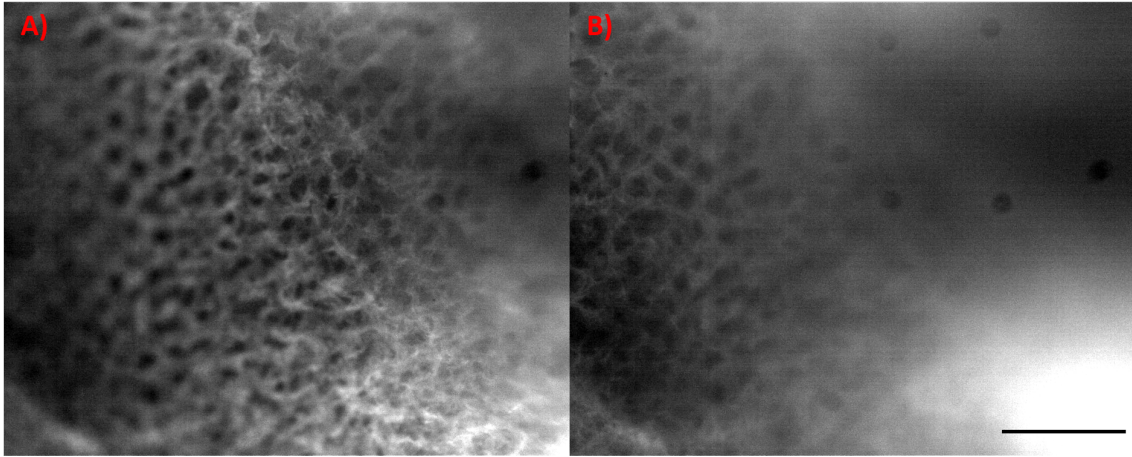


Figure 4.6: Result obtained from set-up with Nikon objective. Distance between images is 100 μm in z-direction. Scalebar indicates 200 μm

Due to the resolve power of the objective, some structures on the liver tissue have become distinguishable. However, again, the desired image quality could not be obtained because of the mismatch of the refractive indices. The line profile analysis for this measurement given in the Figure 4.7.

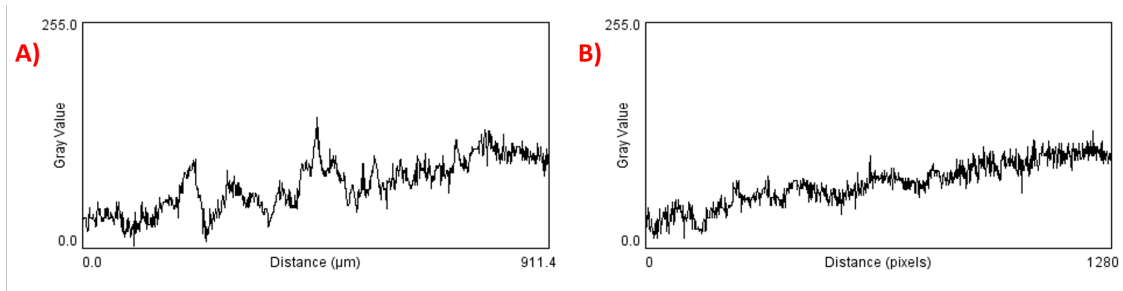


Figure 4.7: The line intensity profile of the second measurement

Although some peaks are more isolated as shown in the figure above, the intensity is the same for most of the image. This situation indicates how sharp the image is. According to results, this image is not a good representation of the tissue.

By using the same configuration, another result (Figure 4.8) and analysis of that result (Figure 4.9) are given below.

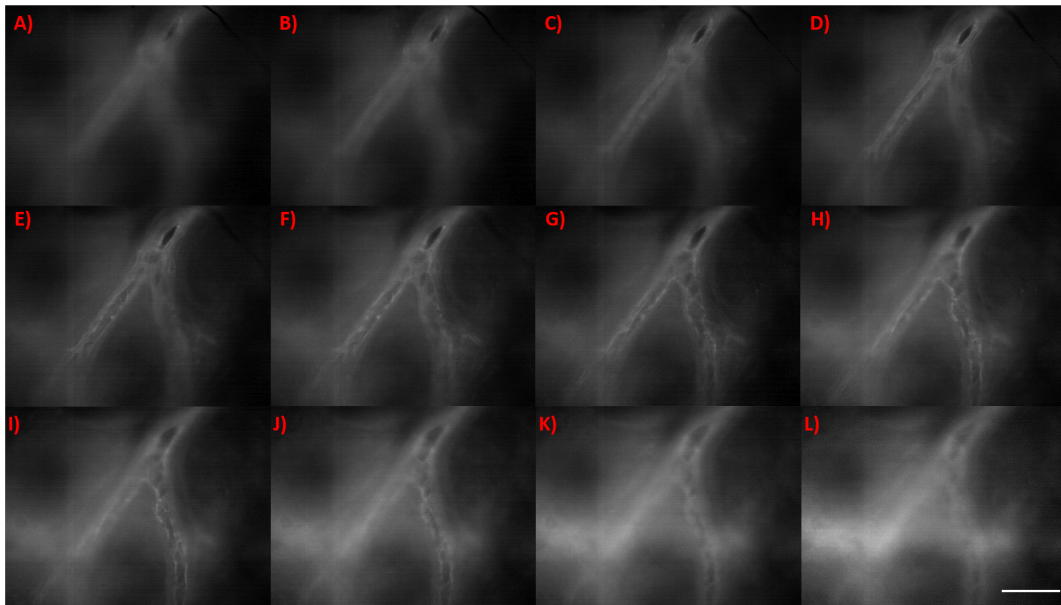


Figure 4.8: Second result obtained from set-up with Nikon objective. Distance between images is 20 μm in z-direction. Scalebar indicates 200 μm

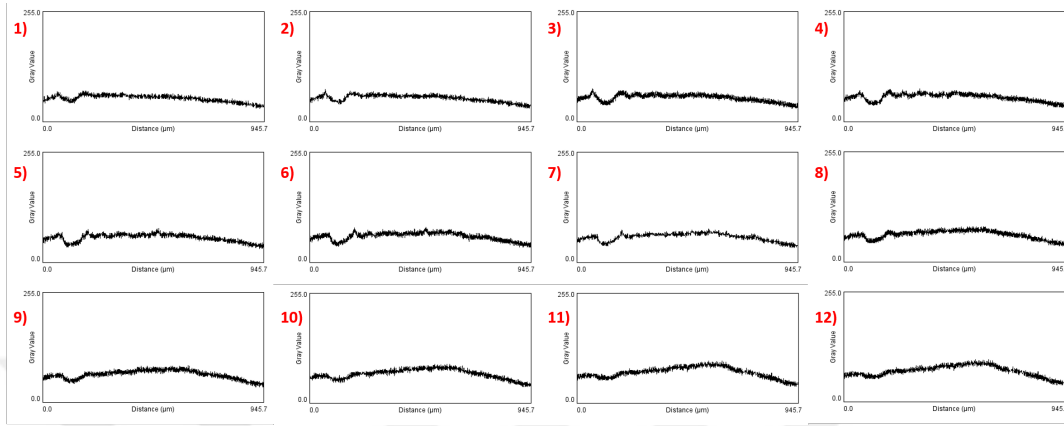


Figure 4.9: The line intensity profile of the second measurement with Nikon objective

Because of the mismatches of the refractive indices, all the images recorded up to this time were not sharp, and in the following experiments the tissue sample was imaged in glycerol medium. To achieve that, a sample chamber which is similar to the one introduced in section 3.3 was used. The holder remained the same. In order to employ the new sample chamber and correct small misalignment, the experimental set-up was rebuilt. Tissue samples first imaged in glycerol medium can be found in the following figure (Figure 4.11). The images has been captured with the Grasshopper3 camera (Figure 4.10).



Figure 4.10: The Stock Photograph of the Grasshopper3 Camera[21]

The specifications of the Grasshopper 3 Camera is given in the following table

(Table 4.4).

Item Number	GS3-U3-23S6C-C
Sensor Type	CMOS
Optical Sensor Format	1/1.2"
Pixel Size	$5.86\mu\text{m} \times 5.86\mu\text{m}$
ADC	10-bit, 12-bit
Frame Rate	163 fps

Table 4.4: Specifications of the Grasshopper3 Camera[21]

In the first experiment in glycerol medium, images were formed by using a matched-pair (1:1).

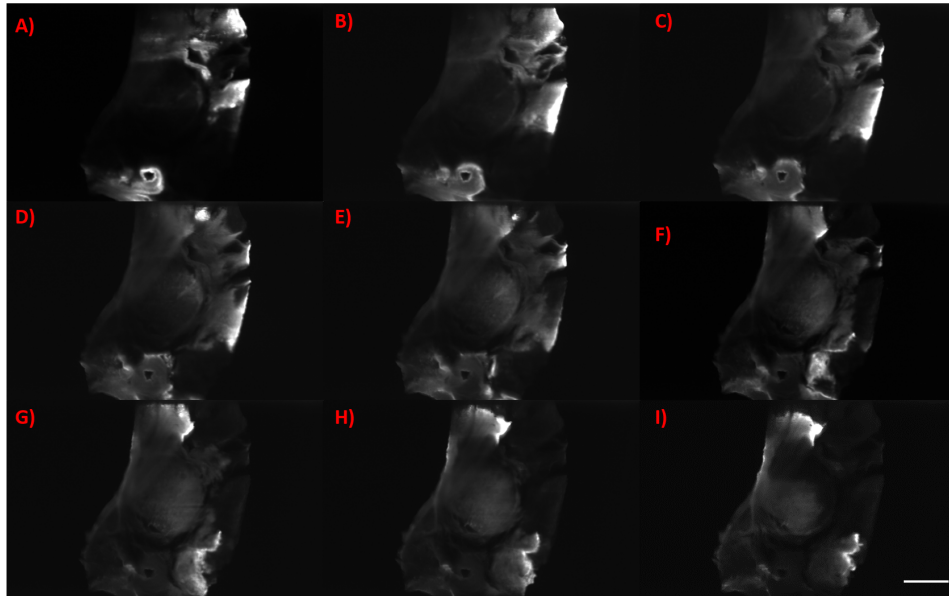


Figure 4.11: In glycerol medium with matched pair (1:1). Distance between images is $15\mu\text{m}$ in z-direction. Scalebar indicates $500\mu\text{m}$

And the line intensity profile for this result can be found as following;

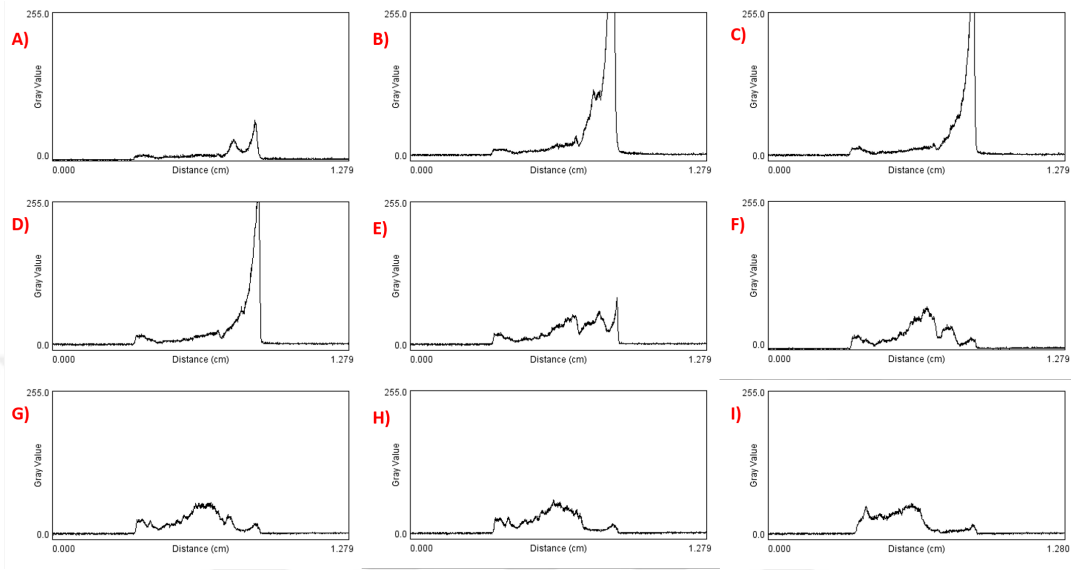


Figure 4.12: Line Intensity Profile of the Measurement given in Figure 4.11

When the two measurements using a matched pair (1:1) are compared (Figure 4.4 and Figure 4.12), the effectiveness of the role of the medium difference on the image quality becomes apparent. In the glycerol medium where the refractive index is compatible with the liver tissue, the structures of the sample become more distinguishable.

At this stage of the study, the illuminating objective was replaced with the best form lens in order to reduce the cost of the microscope. Zemax simulations were performed before selecting the lens for the illumination, detailed information is given in section 3.1.3. In addition to the best form lens, a glycerol dipping objective (CFI Plan Apochromat 10XC Glyc) and a camera (Hamamatsu ORCA-Flash4.0 V3) were employed in the experimental set-up. With these new equipment, the recorded images are expected to be improved.

With the introduction of the new camera (Figure 4.13), the set-up was rebuilt. In this configuration, the orientation detection axis was changed. The holder given in the Figure 3.17 was started to be used from that point of this study. All recorded images of the tissue sample given below were taken in glycerol medium with detection arm includes Hamamatsu ORCA-Flash4.0 V3 and Apochromat 10XC Glyc.



Figure 4.13: The stock photograph of the Hamamatsu ORCA-Flash4.0 V3[22]

The specifications of the camera can be found in the Table 4.5.

Item Number	C13440-20CU
Sensor Type	sCMOS
Dynamic Range	37000:1
Pixel Size	$6.5\mu\text{m} \times 6.5\mu\text{m}$
Pixel Array	2048x2048
Frame Rate	100 fps

Table 4.5: Specifications of the Hamamatsu ORCA-Flash4.0 V3[22]

With the addition of the new lens (Figure 4.14), the refractive index problem was expected to disappear. Because this objective is designed to support multiple refractive indices within the range of 1.33 to 1.51 where the tissue's refractive index was covered.



Figure 4.14: The stock photograph of the CFI Plan Apochromat 10XC Glyc[6]

The specifications of the objective is given in the Table 4.6.

Model	CFI Plan Apochromat 10XC Glyc
Type	Apochromat
M ^a	10X
WD	5.50 mm
Type	Infinity Corrected
NA	0.50

Table 4.6: Specifications of the CFI Plan Apochromat 10XC Glyc[6]

Two of the results obtained with the configuration which is mentioned above are given in the following figure. One of the result contains narrow tissue sample, and the other figure represents wider one. After each figure includes recorded images of the tissue, the line intensity profile analysis is given.

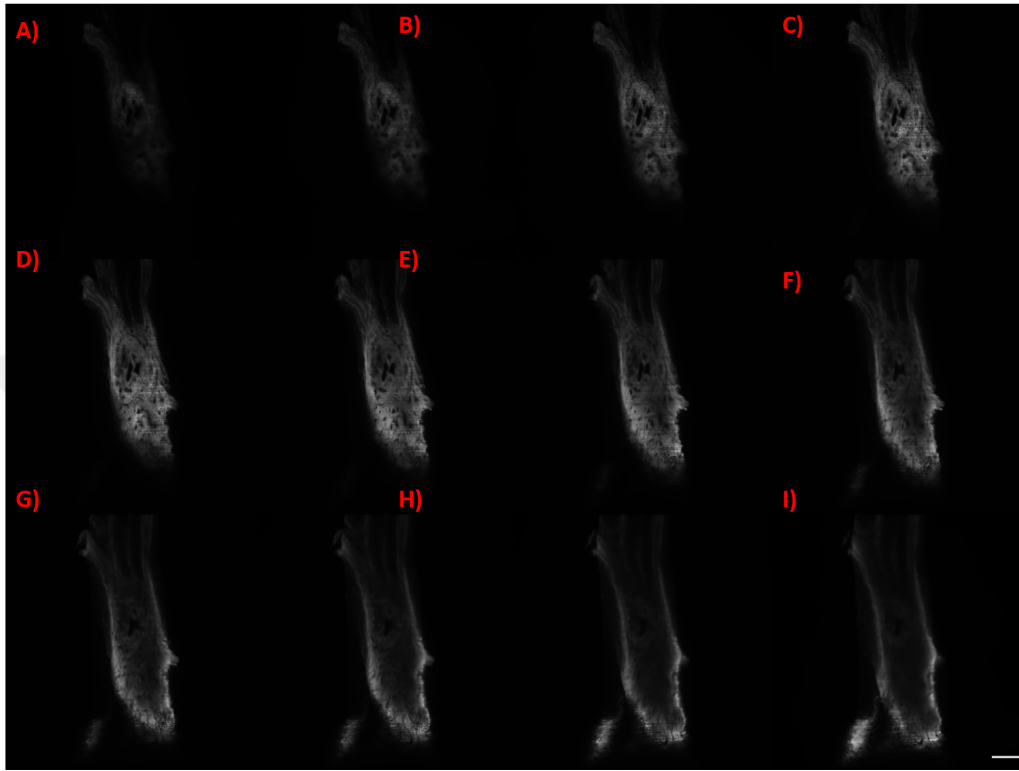


Figure 4.15: Narrow sample, distance between images is $15 \mu\text{m}$ in z-direction. Scalebar indicates $200 \mu\text{m}$

The line intensity profile of the narrow tissue as follows;

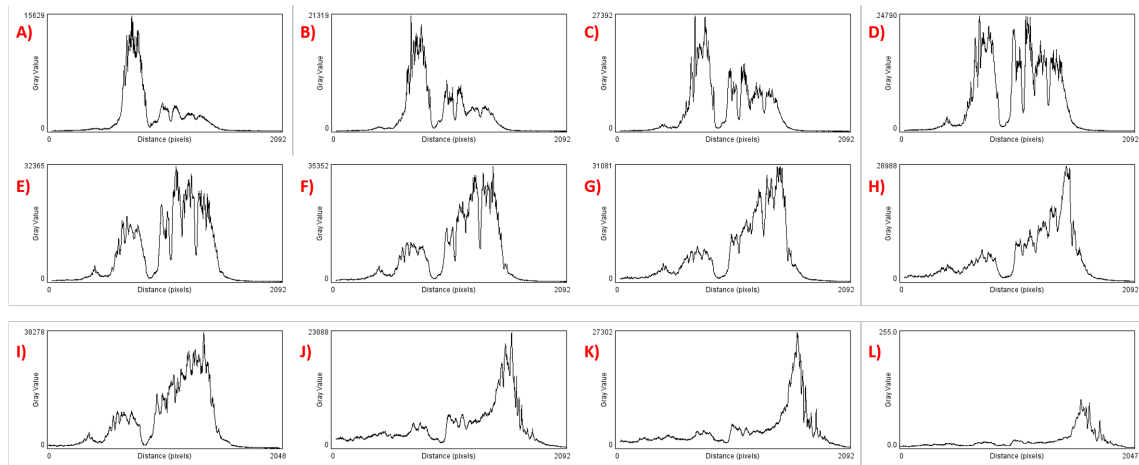


Figure 4.16: The line intensity profile of the narrow tissue

A few problems were encountered later in the study. The first of these problems was that the light sheet scattered in large tissues without illuminating the entire tissue due to one-sided illumination. To overcome this problem, an experimental set-up which has two-sided illumination was built by employing an identical second illumination arm. The following figure (Figure 4.17) contains results which were obtained with the latest sample holder (Figure 3.17) and two-sided illumination.



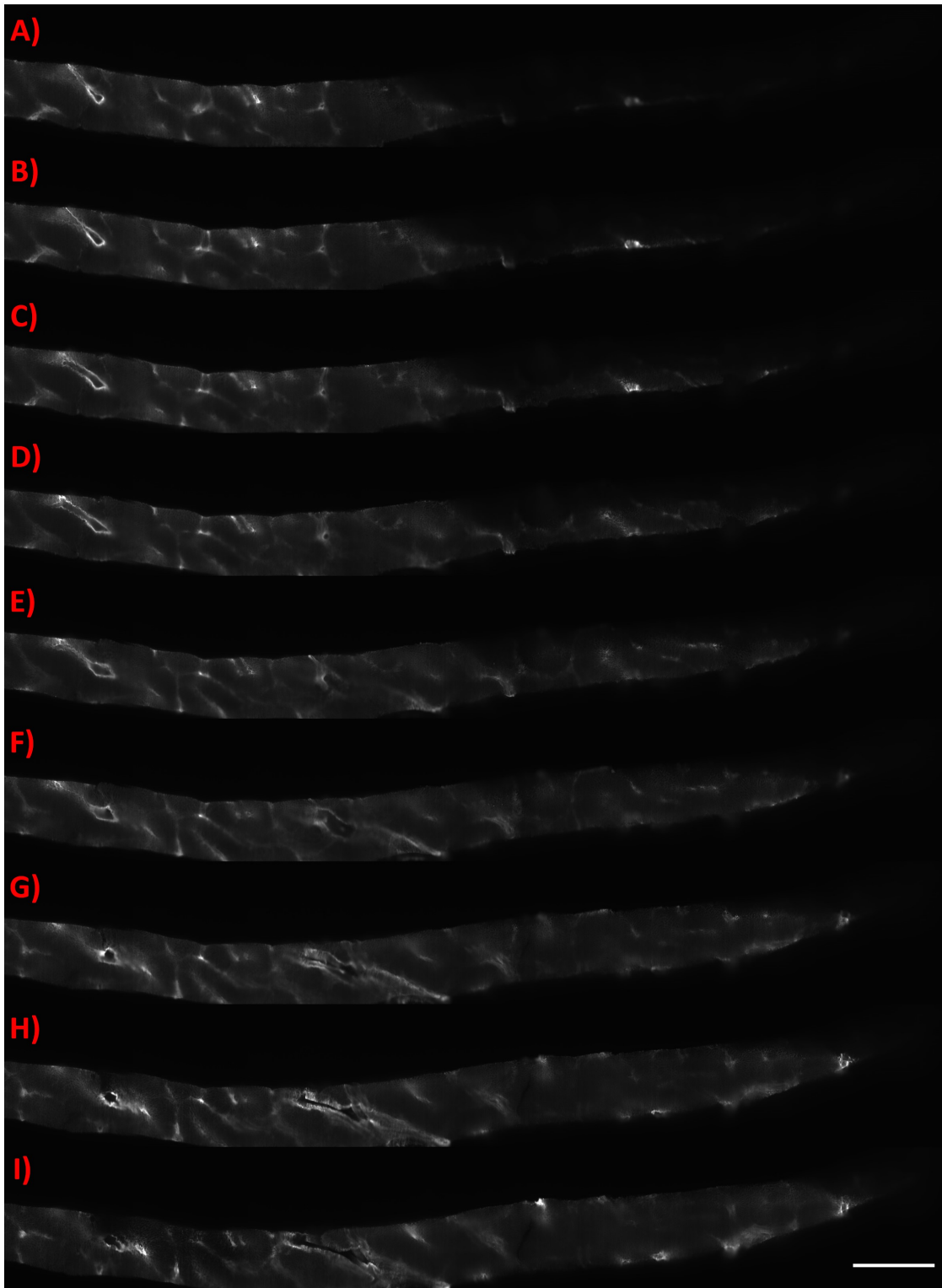


Figure 4.17: Narrow tissue, illuminated two-sided, tiled image, distance between images is $50\ \mu\text{m}$ in z-direction. Scalebar indicates 1 mm

The line intensity profile of the latest results is displayed as,

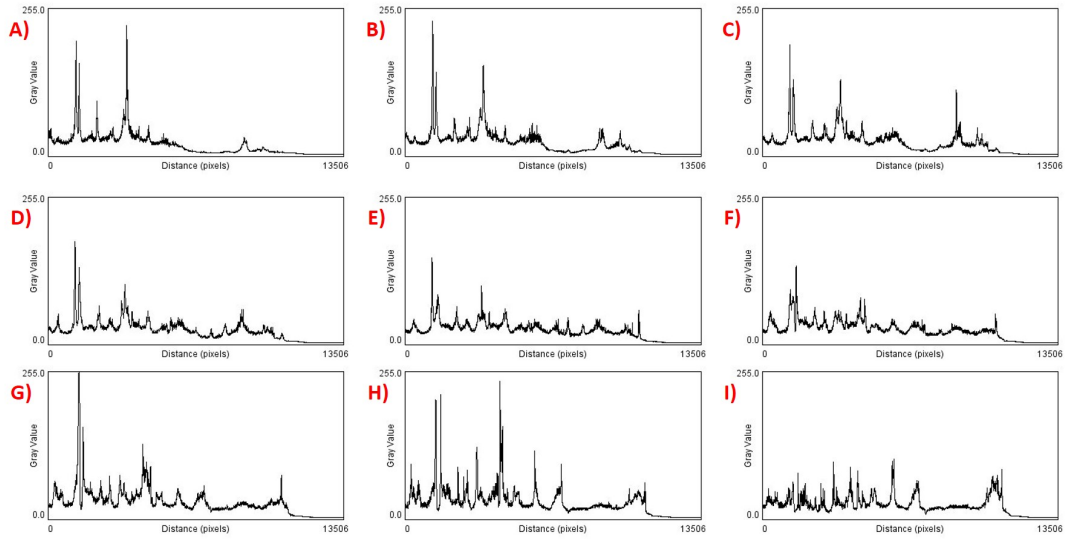


Figure 4.18: The line intensity profile of the narrow tissue, illuminated with two-sided experimental set-up

As it seen in the line intensity profiles, it can be expressed that the quality of the results were improved by analyzing the peaks.

For further quantitative analyses, the boundaries of the tissue sample has crucial role. In order to obtain information about the edges of the sample, scattered image of it was taken by collecting scattered light. The filter was removed and the exposure time was reduced while image recording.

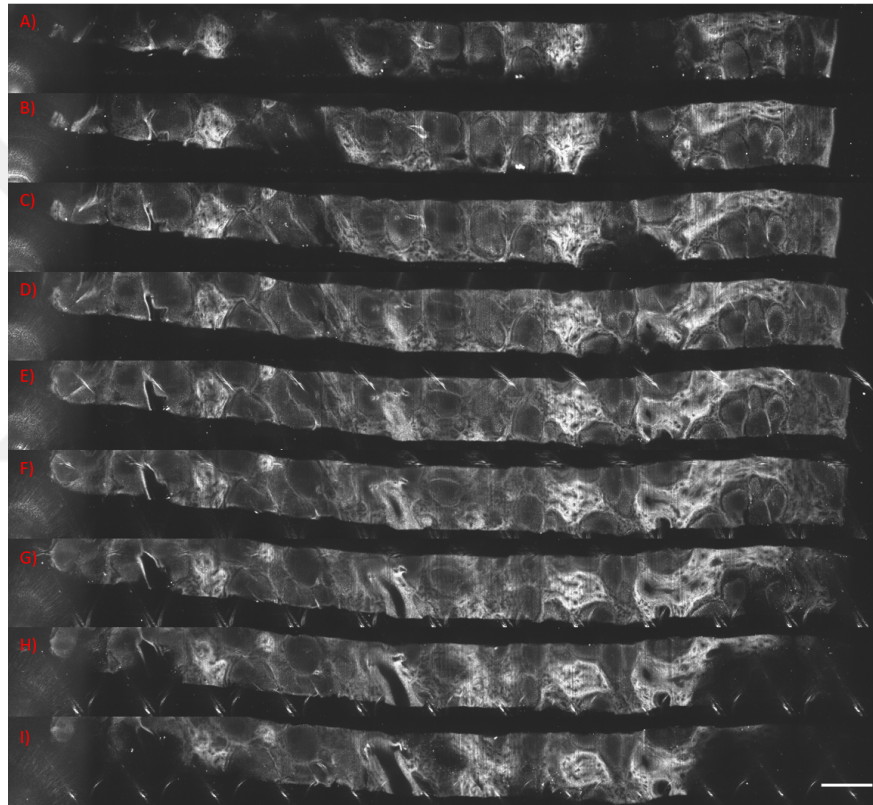


Figure 4.19: Narrow tissue, scattered image, tiled image, distance between images is $50\ \mu\text{m}$ in z-direction. Scalebar indicates 1 mm

Another captured stack image of a liver tissue is given in the following figure.

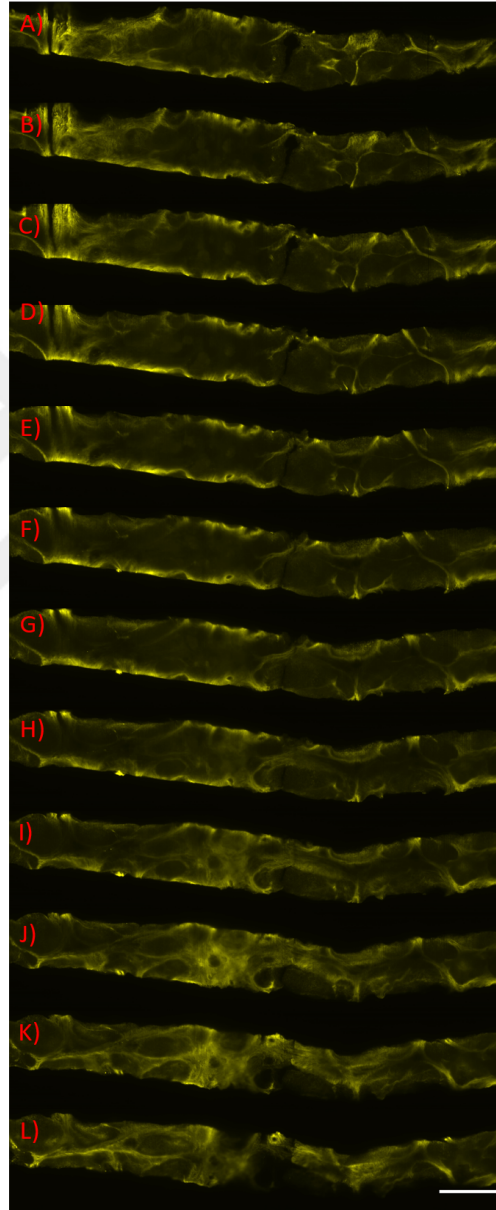


Figure 4.20: Narrow tissue, illuminated one-sided, tiled image, distance between images is $50\ \mu\text{m}$ in z-direction. Scalebar indicates 1 mm

As final result, three-dimensional reconstructed cleared liver tissue sample is given in the following figure. The three-dimensional reconstruction has been obtained by using the 3D Projection plug-in of Fiji ImageJ.

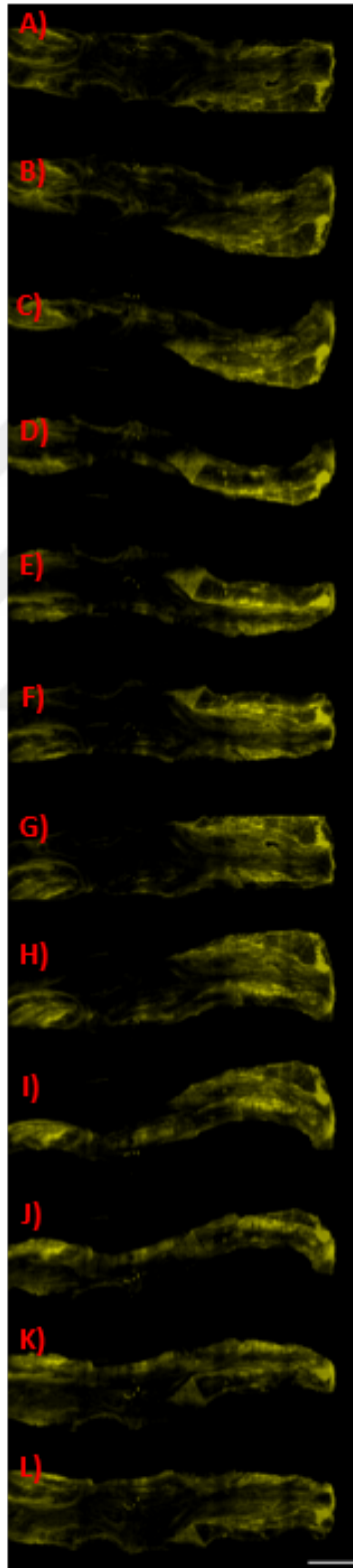


Figure 4.21: The three-dimensional reconstructed tissue sample. Angle between images is 30 degree. Scalebar indicates 1 mm

Chapter 5

CONCLUSION

In this work, an optical set-up was built for cleared tissue imaging. Several experiments have been performed to reach the highest image quality output. During those experiments different optical equipment were used such as objectives and cameras. Besides imaging experiments, two computer programs were developed for measurements. One of them was to calculate the thickness of the light sheet, the other one was for programming the camera.

Two configurations of the optical set-ups were established. First one was SPIM whose unique features are being one-sided light sheet illumination and second one was the ultra-microscope. There are two main differences between them. The orientation of detecting path and the number of illumination arms are those differences. Finalized experimental set-up was developed as ultra-microscope, employing a diode laser which has 525 nm wavelength with 25mW output power, cylindrical lenses, a 3D motorized stage, a sCMOS camera and lots of lenses. Several improvements have taken place which are the replacement of the optical elements for better results and development of a graphical user interface to communicate with the camera for easier and convenient imaging experiments.

In conclusion, a home-built LSFM set-up was developed to image cleared tissues. For this set-up, a custom and user-friendly software was developed. 3D images of the samples were reconstructed by using Fiji ImageJ software. This study has not been finalized, an image processing algorithm to reduce striping and noise and employing better optical components that can improve the set-up are expected in the future works.

Appendix A

MATLAB CODE

```
1 % % % % % % % % % % % % %
2 %  designed by @qmeryaman %
3 % % % % % % % % % % % % %
4 % % 2019 - April - 29 % %
5 % % % % % % % % % % % % %
6
7 clear all;
8 tic
9
10 Files=dir( '*.png' );
11 sheet_thickness = zeros(1,length(Files));
12 for i = (1:length(Files))
13
14 I=imread(Files(i).name);
15
16 blue = I(:,(10:(end-10)),size(I,3));
17 [M,indx]=max(max(blue));
18
19 %When Lightsheet is parallel to the y axis swap the ...
20 ... parameters on row 22 & 23.
21
22 linerow = [indx indx];
23 linecolumn = [0 size(I,2)];
```

```
24 d = improfile(I,linecolumn ,linerow);
25 data=d(:,1 ,size(I,3));
26 [M2,indy]=max(data);
27
28 % Those figures show Intensity profile of Lightsheets.
29
30 % figure
31 % imshow(I)
32 % hold on
33 % plot(linecolumn ,linerow , 'r ')
34 % figure
35 % plot(data , 'b ')
36
37
38 % Width determines the number of data points around center
...
39 ... to fit the plot.
40
41 width=7;
42 y=data(indy-width:indy+width)-min(data);
43 x=linspace(indy-width ,indy+width ,length(y));
44
45
46 [xData , yData] = prepareCurveData( x' , y );
47
48 % Set up fitype and options.
49 ft = fitype( 'gauss1' );
50 opts = fitoptions( 'Method', 'NonlinearLeastSquares' );
51 opts.Display = 'Off';
```

```
52 opts.Lower = [-Inf -Inf 0];
53
54
55 % Fit model to data.
56 [fitresult, gof] = fit( xData, yData, ft, opts );
57
58 % Those figures are to plot fit with data.
59
60 % figure( 'Name', 'untitled fit 1' );
61 % h = plot( fitresult, xData, yData );
62 % legend( h, 'y vs. x', 'untitled fit 1', 'Location', '
    NorthEast' );
63 % % Label axes
64 % xlabel x
65 % ylabel y
66 % title (Files(i).name)
67 % grid on
68
69 c = fitresult.cl;
70 fwhm = c*(2*sqrt(log(2)));
71 sheet_thickness(i) = fwhm*5.86;
72 d_name = Files(i).name;
73 count = length(d_name);
74 if (d_name(1)=='_')
75     dis_name = d_name(2:(end-4));
76     distance(i) = -1*str2double(dis_name);
77 else
78     dis_name = d_name(1:(end-4));
79     distance(i) = str2double(dis_name);
```

```
80 end
81 end
82 close all
83
84 for i=1:length(sheet_thickness)
85     if sheet_thickness(i)==min(sheet_thickness);
86         min_loc=i;
87     end
88 end
89
90 a=strcat('minimum thickness=' , num2str( min(sheet_thickness)
        , '%0.4f' ), 'um');
91 plot(distance , sheet_thickness , 'o')
92 text(min_loc , min(sheet_thickness)-1, a)
93 title 'Plot of Sheet Thickness'
94 xlabel 'distance in z (um)'
95 ylabel 'Sheet Thickness (um)'
96 toc
```

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