ENGINEERING PHYSICS
BACHELOR THESIS

DESIGN, IMPLEMENTATION AND TEST OF A ROUTINE LIGHT SHEET FLUORESCENCE MICROSCOPE

Adrià Escobet Montalbán

Supervised by:
Dr. Jordi Andilla, ICFO
Dr. David Artigas, UPC and ICFO
Dr. Pablo Loza-Alvarez. ICFO

Barcelona, June of 2015
**CONTENTS**

Abstract ................................................................................................................................. 3

1. Introduction .................................................................................................................... 4

  1.1 Fundamentals of Optical Microscopy .............................................................. 4

  1.2 Optical Sectioning ............................................................................................. 5

2. Foundations of Light Sheet Fluorescence Microscopy ............................................. 8

  2.1 Early Days of Light Sheet Fluorescence Microscopy .................................... 8

  2.2 Principle ................................................................................................................ 9

     2.2.1 Illumination .................................................................................................... 9

     2.2.2 Detection ...................................................................................................... 11

  2.3 Implementation ..................................................................................................... 11

  2.4 Sample Mounting ............................................................................................... 14

     2.4.1 Embedded Samples .................................................................................. 14

     2.4.2 Hanging Samples ...................................................................................... 16

     2.4.3 Enclosed Samples ..................................................................................... 16

     2.4.4 Free Samples ............................................................................................ 16

3. Macro SPIM Microscope ............................................................................................. 17

  3.1 Motivation ............................................................................................................ 17

  3.2 Design .................................................................................................................. 17

     3.2.1 Detection Path ............................................................................................. 18

     3.2.2 Illumination Path ......................................................................................... 22

  3.3 Building and Pre-Alignment .............................................................................. 29

     3.3.1 Lasers .......................................................................................................... 29

     3.3.2 Light Sheet Generation ............................................................................. 30

     3.3.3 Chamber ...................................................................................................... 30

     3.3.4 Detection System ....................................................................................... 31

     3.3.5 Diaphragms and Pinholes .......................................................................... 31

  3.4 Light Sheet Characterization and Fine Alignment .............................................. 31

     3.4.1 Mirror at 45° .............................................................................................. 31

     3.4.2 Fluorescent Medium .................................................................................. 33

  3.5 Control Software .................................................................................................... 34

     3.5.1 Timeout ........................................................................................................ 36

     3.5.2 Settings ........................................................................................................ 36

     3.5.3 Define Home .............................................................................................. 36

     3.5.4 Go Home ................................................................................................... 36
ABSTRACT

Microscopy has revealed an astonishing new world that was previously invisible to our eyes. Unfortunately, we still have very little knowledge of how life actually works, and for that reason new powerful microscopy techniques have to be developed. The Super Resolution Light Microscopy & Nanoscopy Facility (SLN) led by Dr. Pablo Loza-Alvarez at The Institute of Photonic Sciences (ICFO) is equipped with front-end microscopy techniques that are able to operate a step beyond the commercial state of the art. However, there is a need to incorporate a microscope capable of acquiring fast three-dimensional images of large microscopic samples. One option to fulfil this need, is the implementation of a new light sheet fluorescence microscope, which must be robust and user-friendly for non-specialist users.

Light sheet fluorescence microscopy is a relatively new technique that has grown in popularity in the life sciences since its invention, becoming eventually the Nature Method of the Year 2014. Still, it has a great potential to keep advancing. Its ability to image many cells simultaneously within large samples, opens up the possibility of imaging whole organs of small animals such as living hearts, developing embryos and functioning brains. Understanding the global and mysterious properties of the human brain is one of the greatest challenges facing 21st century science, and light sheet fluorescence microscopy can play an important part in it. This technique has already become one of the major imaging tools used to produce dynamic pictures of the brain in The White House BRAIN Initiative, which is aimed at the understanding and cure of devastating diseases such as Alzheimer, Parkinson and Epilepsy.

In this work, I will describe the design, implementation and test of the routine light sheet fluorescence microscope that I built in the SLN Facility at ICFO. It is designed to image entire clarified mouse brains but also to provide enough flexibility to image a wide range of large biological samples.
1- INTRODUCTION

1.1- FUNDAMENTALS OF OPTICAL MICROSCOPY

Microscopes have been used since the 17th century to view and analyse objects or details of objects that cannot be seen with the naked eye. There are three well-known branches of microscopy: optical, electron, and scanning probe microscopy. Here, we focus on optical microscopy. Optical or light microscopy involves passing visible light transmitted through, reflected from or emitted by the sample through a single or multiple lenses to allow a magnified view of the sample (Fig. 1, A). The resulting image can be detected directly by the eye, imaged on a photographic plate or captured digitally by a camera. The portion of the sample that is imaged is called the Field Of View (FOV) which is determined by the magnification of the optical system and the sensor size. The magnification of an infinity corrected microscope is given by:

\[ M = \frac{f_{TL}}{f_{obj}} \]  

(1)

The resolution of an optical system is measured as the size of its Point Spread Function (PSF), which is the three-dimensional (3D) image that a point source of light would produce. The image of a microscope is generated by the convolution of the object with the PSF of the system (Fig. 1, B) and, therefore, it is convenient to have a PSF as small as possible. PSF’s dimensions are dictated by the numerical aperture of the detection objective. The Numerical Aperture (NA) of a lens measures its ability to gather light and it is related to the maximum collection angle of sample’s light by the objective (Eq. 2). Thus, the larger the NA is, the smaller the PSF becomes and, consequently, the higher the resolution can be.

\[ NA = n \sin \theta \]  

(2)

Image quality, however, is not only determined by the resolution of the optical system but also by the contrast mechanism used to generate the image. Some of the most common mechanisms to increase contrast in both stained and unstained samples are based on absorbance of light in the sample (bright field microscopy), scattering of light by the sample (dark field microscopy), interference of different path lengths of light through the sample (phase contrast microscopy) or fluorescently labelling samples (fluorescence microscopy). In fluorescence microscopy (Fig. 2, B) the specimen is illuminated with light of a certain wavelength which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (Stokes shift). One of the most commonly used fluorophores are fluorescent proteins such as Green
Fluorescent Protein (GFP) and Yellow Fluorescent Protein (YFP). In general, the more intense the excitation illumination is, the higher the signal emitted by the sample is, however, high power illumination conditions can increase the probability of non-radiative relaxations such as photobleaching or quenching, reducing the total amount of light emitted by the sample (Fig. 2 A). Therefore, the user always has to find a trade-off between signal from the sample and photobleaching. One of the simplest fluorescence microscopes are the so-called widefield epifluorescence microscopes, in which excitation and detection are done through the same light path. In such configuration, excitation and fluorescently emitted light are separated by a dichroic mirror. The performance of epifluorescence microscopes is drastically dropped in three-dimensional imaging because the entire sample is illuminated in each acquisition generating out-of-focus light that blurs the image. Therefore, optical sectioning is essential to acquire 3D images of large microscopic samples and requires its own dedicated section.

![Figure 2. A: Jablonski diagram of light absorption and relaxation processes. B: Alexa Fluor 555 absorption and emission spectra. Extracted from http://www.olympusmicro.com and http://www.microscopyu.com/ respectively.](image)

### 1.2- OPTICAL SECTIONING

The 3D extent of a fixed sample can be imaged at high resolution by physically sectioning it. The achievable resolution is determined by the thickness of the sections and the imaging modality that is used to image them. However, physical sectioning limits studies in biology because it requires samples to be fixed. Hence, other strategies as optical sectioning are required for 3D fluorescence microscopy.

In contrast to mechanical sectioning, optical sectioning makes it possible to image individual planes of a three-dimensional sample while keeping it alive and minimally damaged. Light is confined in a well-defined section with a certain thickness and an image can be extracted without the blur coming from different depths inside the sample. Optical sectioning in fluorescence microscopy is usually achieved in three different ways: (1) image processing, for example deconvolution [e.g. McNally et al., 1999]; (2) illuminating the whole sample and rejecting unwanted light; or (3) restricting the fluorescence excitation to a single plane of interest and detecting all the fluorescence from the sample.
Optical sectioning by image processing can be achieved by simply processing images obtained with any standard widefield microscope. In widefield imaging, as the whole sample is illuminated, all fluorescently labelled structures emit light, irrespective of whether they are in focus or not. So the image of a certain structure is always blurred by the contribution of light from structures that are out of focus, resulting in a loss of contrast. However, as explained in section 1.1, blurring is not caused by a random process but can be well defined by the PSF. Since the final image is the result of the convolution of the object and the PSF of the optical system, knowing the exact PSF can be used to reverse the process and deconvolve the image using computational algorithms. The final result after deconvolution is a 3D stack of clearer images in which the blurry contribution from adjacent planes has been suppressed. Nevertheless, deconvolution relies on a number of assumptions about the optical properties of the sample and about the performance of the microscope which in less than ideal conditions can lead to artifacts. Another image processing technique is tomography, for example Optical Projection Tomography (OPT), in which after gathering projection data from multiple directions, the three-dimensional object is reconstructed in the computer.

The second approach to optical sectioning in which the whole sample is illuminated is the Laser Scanning Confocal Microscopy (LSCM) [Pawley, 2006]. In LSCM, an image of a full plane is built-up point-by-point by raster scanning a laser to cover the entire surface. All depths of the sample are illuminated but a pinhole rejects out-of-focus light coming from unwanted regions and only keeps the light from a single plane (Fig. 3). The confocal microscope is ideally suited for the 3D imaging of samples of thickness of the order of 100 µm and has become a routine instrument found in many laboratories. However, its performance in live imaging is mainly limited by acquisition time. As each image is obtained by combining the information of many points, the acquisition speed becomes very slow and it cannot follow fast biological processes. The scanning speed of the laser can be increased but, as a result, fewer fluorescence photons are emitted resulting in worse signal to noise ratio in the images. Another important drawback of this technique is high photo-bleaching and photodamage since the whole volume of the sample is illuminated multiple times in a 3D stack acquisition. Such limitations can be overcome by designing an improved scanning system, this is the case of Spinning-Disk Confocal Microscopy [Nakano, 2002].

Figure 3. Schematic representation of a LSCM. Adapted from http://zeiss-campus.magnet.fsu.edu/
Finally, in the third approach to optical sectioning, fluorescence excitation only takes place in the imaged plane leaving the rest of the sample unperturbed. Therefore, photodamage can be reduced to a minimum. This approach is taken in two-photon microscopy and in light sheet fluorescence microscopy in two very different ways. Maria Göppert-Mayer established the theoretical basis of two-photon excitation in 1931 but it was not until 1963 when this photophysical effect was experimentally verified by Kaiser and Garret in 1963. As So stated in 2002 [So, 2002], two-photon excitation is a fluorescence process in which a fluorophore is excited by the simultaneous absorption of two photons. The familiar one-photon fluorescence process involves exciting a fluorophore from the electronic ground state to an excited state by a single photon. However, the same excitation process can be generated by the simultaneous absorption of two less energetic photons under sufficiently intense laser illumination (Fig. 4). This nonlinear process can occur if the sum of the energies of the two photons (typically in the infrared spectral range) is greater than the energy gap between the molecule’s ground and excited states. For example, a fluorophore that normally absorbs ultraviolet light (350 nm) can also be excited by about two photons of near-infrared light (700 nm). Since the process relies on the absorption of two photons at the same time, the probability of two-photon excitation scales as the square of the excitation radiance. So, in order to produce a significant number of two-photon absorption events, the photon density must be some orders of magnitude higher than the required to generate the same number of one-photon absorptions. Such high photon density is achieved by focusing ultra-short pulsed lasers, in which the power during the peak of the pulse is extremely high. The main advantage of this technique is that the laser focal point is the only location along the optical path where the photon density is high enough to generate significant two-photon excitation. Thus, this nonlinear phenomenon prevents excitation outside of the focal plane obtaining intrinsic optical sectioning. Moreover, the use of longer wavelengths increases the penetration depth (up to 700 μm) but reduces the resolution compared to single-photon confocal microscopy. In addition, since high power laser pulses are required, the whole sample can suffer from the detrimental effects of light, such as heating, photobleaching and photodamage. On the other hand, in light sheet fluorescence microscopy a different approach is taken to selectively illuminate the area of interest while at the same time preventing the sample from damage. LSFM is the central core technology of this work and it is deeply described in the following chapter.

Figure 4. A: Single-photon excitation. B: Two photon excitation. Adapted from Zipfel et al., 2003.
2- FOUNDATIONS OF LIGHT SHEET FLUORESCENCE MICROSCOPY

2.1- EARLY DAYS OF LIGHT SHEET MICROSCOPY

The first device that employed the principle of light sheet microscopy was described by Siedentopf and Zsigmondy in 1903 [Siedentopf and Zsigmondy, 1903]. They observed gold particles in glasses by projecting a light sheet made of sunlight on the sample (Fig. 5). The light sheet allowed them to focus on a few particles in an ensemble of many particles. The use of a light sheet instead of a uniform illumination dramatically increases the contrast and makes visible single particles, which would otherwise be indistinguishable from the rest. Modern applications of this idea include the Thin Light Sheet Microscope (TLSM) for visualizing aquatic microbes [Fuchs et al., 2002] and the single particle tracking microscope [Ritter et al., 2008].

Later, light sheets were used for extended depth of field photography [Zampol, 1960], deep focus microscopy [Simon, 1965; McLachlan, 1968] and 3D light scanning macrography [Huber et al., 2001]. In this application, a macroscopic object is slowly moved through a light sheet, while a camera that is focused on the illuminated plane is continuously exposed. The result is a picture of the object in which every portion is in focus.

In 1993, the first light sheets were used to illuminate macroscopic biological samples labelled with fluorescent dyes to obtain optical sectioning. Voie and co-workers developed a light sheet microscope system, called Orthogonal Plane Fluorescence Optical Sectioning (OPFOS) to image the internal architecture of the cochlea [Voie et al., 1993]. OPFOS featured all of the elements that are present in current Light Sheet Fluorescence Microscopy (LSFM) devices: laser, beam expander, cylindrical lens, specimen chamber, orthogonal illumination and specimen movement for z-stack creation. Although Voie and colleagues published several articles in widely read journals, it was not until the publication of the Selective Plane Illumination Microscopy (SPIM) paper in 2004 in Science [Huisken et al. 2004] that the development and use of LSFM microscopes were accelerated. This article highlighted the usefulness of LSFM for investigating embryonic development including imaging of heartbeats, GFP tagged ganglion cells in live medaka embryos (Oryzias latipes) and embryogenesis of a Drosophila melanogaster embryo over a 17-hr period.

Figure 5. The first light sheet microscope built in 1903. Adapted from Siedentopf and Zsigmondy, 1903.
2.2- **PRINCIPLE**

Light Sheet Fluorescence Microscopy (LSFM) combines two separate orthogonal optical paths, one for fast widefield detection and one for illumination with a thin sheet of light (Fig. 6, A). Three-dimensional stacks of images are acquired by stepwise movement of the sample along the axis of the detection lens.

![Figure 6. Principle of LSFM. A: Uncoupled illumination and detection paths. B: Schematic representation of the light sheet and FOV. Adapted from Huisken et al., 2009, and Weber et al., 2014, respectively.](image)

The following sections describe LSFM’s unique uncoupled illumination-detection configuration which addresses three fundamental limitations of other fluorescence microscopes: optical sectioning, phototoxicity and acquisition speed. They are based mainly on the work of Huisken and Weber [Huisken, (2009) and Weber (2014)].

2.2.1- **Illumination**

In LSFM, a single plane of the sample is illuminated with a very thin sheet of light. The centre of the light sheet is set to coincide with the focal plane of the detection objective. As the reader can infer, this unique illumination geometry efficiently reduces the energy input with respect to LSCM (Fig. 8). In addition, as each plane is only exposed once during a stack acquisition, photobleaching and phototoxicity are reduced to a minimum. The thickness of the light sheet determines the axial size of the optical slice and is much thinner than the depth of field of the detection objective. With LSFM, one can therefore acquire thin optical sections across large FOVs in big specimens.

The properties of the light sheet such as thickness, uniformity, position and ability to penetrate scattering media, determine the performance of the microscope. The light sheet must be aligned with the focal plane of the detection path and its waist positioned in the centre of the FOV (Fig. 6, B). Unfortunately, the effect of diffraction strongly modifies its dimensions making the thickness change across the field of view. Therefore, the NA of the illumination needs to be chosen carefully to generate a light sheet that is sufficiently thin across the entire FOV. Due to the diffraction-limited shape of the light sheet, one can generally choose between a thin light sheet for small FOVs and thicker light sheets for large FOVs (Fig. 7, A).
Fundamentally, there exist two ways of generating the light sheet (Fig. 7, B). The most straightforward approach is to create a static light sheet with a cylindrical lens. The light of the laser is expanded and a cylindrical lens focuses it in only one dimension generating a thin sheet of light. Alternatively, a virtual light sheet can be generated by scanning a single intense laser beam rapidly up and down. This concept was introduced as digital scanned laser light sheet fluorescence microscopy (DSLM) [Keller et al., 2008] in some of its various modalities: Gaussian beam, two-photon Gaussian beam and two-photon Bessel beam. Figures adapted from Huisken et al., 2014, and Huisken, 2012, respectively.

Figure 7. Left: Schematic representation of the relation between light sheet thickness and field of view. Right: Different ways of generating a light sheet. The first is a static light sheet generated by a cylindrical lens. The other three correspond to digital scanned laser light sheet fluorescence microscopy (DSLM) [Keller et al., 2008] in some of its various modalities: Gaussian beam, two-photon Gaussian beam and two-photon Bessel beam. Figures adapted from Huisken et al., 2014, and Huisken, 2012, respectively.

Figure 8. Image acquisition process in LSCM (A, B) and in LSFM (C, D). Illumination and detection mechanisms are split. Adapted from Huisken et al., 2009.
2.2.2- Detection

The detection system in LSFM generally consists of a widefield microscope. A detection objective collects light from its focal plane and passes it through a fluorescence filter, then, a tube lens projects the light onto a camera. As described in section 1.2, confocal microscopy is inherently slow because of the need to scan the laser, alternatively, optical sectioning in LSFM is achieved with just a single exposure (Fig. 8). A Charge-Coupled Device (CDD) camera collects all the fluorescence from the sample at once without the need for raster scanning (Fig. 9, A and B). The intrinsic optical sectioning and speed of light sheet microscopy, makes it an ideal technique to image fast dynamic developmental processes in sensitive living specimens in a minimally invasive manner.

Theoretically, the lateral resolution in LSFM is equivalent to the lateral resolution in widefield microscopy, which is determined by the NA of the objective lens and the wavelength of the fluorophore. In practice, however, the lateral resolution in LSFM is often even better because of improved contrast due to the elimination of out-of-focus haze by intrinsic optical sectioning. The axial resolution is simply given by the light sheet thickness. In the case of imaging large samples with low-NA, low-magnification, and long-working-distance objectives, one can obtain remarkably better axial resolution than widefield and confocal microscopes. For example, using a 0.17 NA objective, one obtains a 32.4 µm axial resolution with the widefield microscope, 25.6 with the confocal and just 10.4 µm with a LSFM, still having an acceptable FOV.

![Figure 9. A: Point scanning detection in LSCM. B: Full-frame detection in LSFM. C and D: Comparison of the resolution performance of widefield, confocal and LSFM microscopes. Figures adapted from Weber et al., 2014.]

2.3- IMPLEMENTATION

Surprisingly, microscope designs have not changed much over the past decades: even though the technical details of the microscopes have changed, it seems as if the ergonomics of the microscope, especially the ability to view the sample by eye, have always been very important. But things have changed since the early days of microscopy. We can leave behind
the antiquated designs and come up with novel implementations to maximize image quality and sample preservation. To accomplish the orthogonal optical arrangement of the two beam paths, light sheet microscopes are often set up in the horizontal plane, with the sample either hanging from above or resting in the chamber at the intersection of illumination and detection paths.

Since the invention of light sheet fluorescence microscopy, there has been a tremendous effort to improve the technique. Each investigator that has developed a somewhat different system has given it a different name and acronym\(^1\), being LSFM the most general and accepted one (Table 1). As opposed to traditional microscopes in which the sample had to have a particular size and optical properties, in light sheet microscopy the microscope is adapted to the specimen. Therefore, the sample can be kept under ideal conditions while still achieving high spatiotemporal resolution. Consequently, depending on the sample of interest, light sheet microscopes may look very different even though they still share the same fundamental principle (Fig. 10).

![Figure 10. Schematic representations of various fluorescence microscopy techniques. From left to right: Epifluorescence, Selective Plane Illumination Microscopy (SPIM), multidirectional SPIM (mSPIM) and Ultramicroscope. Adapted from Huisken and Stainier, 2009.](image)

One of the most relevant LSFM implementations is the Selective Plane Illumination Microscope (SPIM) developed by Jan Huisken [Huisken et al., 2004]. It consists of a single-sided illumination device in which the light sheet is generated by a cylindrical lens. It allows sample rotation about the vertical axis to obtain a better 3D image. Due to the illumination with a collimated light sheet from the side, any obstacle in the beam path will cast a shadow along the direction of illumination and will become visible as a stripe in the image. Moreover, scattering broadens and absorption attenuates the light sheet as it traverses the specimen. The resulting images may appear blurry, out of focus and stripy. In this case, a second illumination arm can improve image quality. Its optical configuration is typically identical to the first illumination arm and illuminates the sample from the opposite direction through an additional illumination lens. The light sheets generated by both illumination arms are aligned to coincide with precision in the focal plane of the detection objective. The well-illuminated parts of the images from each illumination side can be then stitched resulting in a final image of evenly good quality (Fig. 11). This is the case of Ultramicroscopy [Dodt et al., 2007]. The Ultramicroscope is capable of imaging large specimens such as a cleared mouse brains without losing image quality along the sample. Another dual-sided illumination LSFM is the

\(^1\) Visit [http://openspim.org/Acronyms](http://openspim.org/Acronyms) to find an updated list of all light sheet microscopy acronyms.
multidirectional SPIM (mSPIM) which not only illuminates the sample from both sides but also adds pivoting to the cylindrical lenses reducing even more the presence of shadows.

![Figure 11. Stitching of two images taken from two different illumination arms. Figure taken Huisken, 2012.](image)

<table>
<thead>
<tr>
<th>Technique</th>
<th>Unique features</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Ideal application</th>
<th>Invention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-photon excitation microscope</td>
<td>True 3D imaging, infrared laser.</td>
<td>Low scattering, high penetration,</td>
<td>Slow scanning, low resolution,</td>
<td>Deep penetration in scattering</td>
<td>Denk et al., 1990.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>optical sectioning, reduced photobleaching.</td>
<td>high cost, high power laser.</td>
<td>tissue.</td>
<td></td>
</tr>
<tr>
<td>Ultramicroscope</td>
<td>Dual-sided light sheet illumination, upright detection path.</td>
<td>Like SPIM, sealed chamber with clearing solution.</td>
<td>No index-matched optics.</td>
<td>Large fixed and cleared specimens.</td>
<td>Dodt et al., 2007.</td>
</tr>
<tr>
<td>DSM</td>
<td>Like SPIM, light sheet generated by beam scanning.</td>
<td>Uniform and adjustable light sheet.</td>
<td>Like SPIM.</td>
<td>Like SPIM.</td>
<td>Keller et al., 2008.</td>
</tr>
</tbody>
</table>

Table 1. Comparison between traditional fluorescence microscopy techniques and some of the most representative light sheet fluorescence microscopes.
2.4- SAMPLE MOUNTING

Any microscopy technique will only be truly useful when it is compatible with biological samples; hence, the mounting of the sample is crucial. In this section, we describe all different sample mounting techniques for light sheet fluorescence microscopy. The following paragraphs are based on the work done by Reynaud and Flood [Reynaud (2015), Flood (2013)].

Traditional microscopes have dictated how an ideal sample should be: thin, flat and transparent. However, biological samples come in a wide range of sizes and their optical properties vary dramatically. LSFM offers the possibility of designing microscopes for specific samples and this can mean doing away with the coverslip and, with it, many well-established protocols for sample mounting.

Each particular implementation of LSFM needs its unique sample preparation protocol. For example, if the setup is horizontal (e.g. SPIM), the sample is usually presented from above, hanging along the gravitation axis, whereas in vertical configuration (e.g. Ultramicroscope) the simplest way is to place the sample on a cuvette filled with medium underneath the objective.

Before entering into detail about the different holding methods, it is important to consider all the possible immersion mediums. Since LSFM allows imaging of either live or fixed samples, the immersion medium must be chosen according to their needs. For living samples it is important to be immersed in an aqueous solution that mimics as much as possible their original environment. In some advanced setups the chamber can be connected to a computer-controlled perfusion pump and an in-line heater which provide temperature-controlled fresh medium. On the other hand, fixed samples allow more flexibility and can sometimes be simply immersed in water. However, some large scattering samples, like mouse brains, require a clarification treatment\(^2\) and an immersion in a clearing solution in order not to lose transparency. And of course, LSFM is not limited to samples immersed in a medium; setups with air lenses can image a sample as it is.

Once the different immersion mediums are known, the features of the most used methods of sample mounting can be presented.

2.4.1- Embedded Samples

Embedding objects in plastic materials is a routine procedure widely used in the preparation of samples for electron microscopy. However, LSFM samples have to be immobilized in a way that their biological activity is not disturbed. Furthermore, transparency of the mounting medium is essential to allow imaging. A basic technique of mounting objects for LSFM is to shape them into a cylinder gel that can then be mounted on a dedicated holder and immersed in the chamber. The cylinder is the best option when the sample is rotated because it minimizes the deformation of the image.

\(^2\) Clarification is process in which brain tissue is made transparent by stripping away lipids that normally block the passage of light and replacing them by a hydrogel scaffold which maintains the original neuronal and protein structure of the brain [Chung & Deisseroth, 2013].
One of the most widely used mounting protocols involves low-melting agarose\(^3\) in a glass capillary (Fig. 12). Here, the specimen is embedded in a solid cylinder of agarose, which is extruded into the medium-filled chamber. The transparent agarose matches the refractive index of water and biological tissue, and under appropriate concentrations it provides enough mechanical stability to move the sample.

Depending on the agarose concentration, two types of embedding can be distinguished: hard embedding and soft embedding. In hard embedding 1.5% or 2% agarose is used to create a solid agarose block which sustains firmly the specimen. However, in the case of imaging living embryos, a softer embedding is required to allow a proper three-dimensional growth of the specimen and 0.1% agarose is used (Fig. 13). Unfortunately such low agarose concentrations are not solid enough to maintain their shape during long term imaging and require an extra external support. Recently some researchers [Weber et al., 2014] have used a tube made of optically clear polymer called Fluorinated Ethylene Propylene (FEP) which has an index of refraction of 1.338 and matches water’s (1.333) much better than typically used glass capillaries (1.52). This way, the specimen can grow more freely and can be imaged without much perturbation.

\(^3\) Agarose is a complex carbohydrate polymer material, generally extracted from seaweed. The molecules are extremely water-soluble due to their large number of hydroxyl groups, and solutions tend to be low-melting point aqueous gels. For example the low melting type Agarose Type VII has a gelling temperature below 30°C and a melting temperature of about 65°C.
2.4.2- **Hanging Samples**

Perhaps the most simple and intuitive way of holding a sample is by simply taking it as it is and placing it in front of the objective. This simple approach can be performed in LSFM by using a simple hook made of glass, stainless steel or plastic to hold large samples such as individual organs (the brain) or complete organisms (insects, fish) (Fig. 14, A). The main disadvantage is that the sample is in most cases partially damaged by the hook.

2.4.3- **Enclosed Samples**

Another important technique of holding samples is to create a container that can hold the object in front of the objective lens (Fig. 14, B). This technique is particularly suitable for specimens that should not be embedded or that need to be constantly maintained in a specific buffer. The container must be basically transparent and it can be easily moulded using a gelling agent specifically chosen for its stiffness and transparency.

![Figure 14. A: Different ways of hanging a sample B: Making and mounting process of an agarose incubation camber. Images extracted from ZEISS Lightsheet Z.1 Sample Preparation, 2013.](image)

2.4.4- **Free Samples**

And finally, the most straightforward method is to simply put the sample inside the chamber resting on its bottom. This method is usually employed with large samples such as mouse brains which need to be immersed in a clearing solution or in non-horizontal configurations such as the Ultramicroscope.
3- MACRO SPIM MICROSCOPE

3.1- MOTIVATION

In this chapter I will describe the design and implementation of the routine light sheet fluorescence microscope that I built in the Super Resolution Light Microscopy & Nanoscopy Facility (SLN) led by Dr. Pablo Loza-Alvarez at The Institute of Photonic Sciences (ICFO). Researchers at the SLN Facility perform continuous R&D in most of the advanced light microscopy techniques and provide access and training to all types of users in the forefront of microscopy for the most demanding biomedical applications. It is open to external collaborations with industry, research centres and Universities.

The SLN Facility contains many different microscopes (LSCM, multiphoton excitation microscope, SPIM, DSLM and STED) but none of them offer fast three dimensional imaging of large samples. Therefore, to fill this gap a new LSFM microscope is needed. Initially the microscope will be adapted to a collaborative project which requires a microscope capable of imaging clarified mouse brains labelled with Venus\(^4\) fluorophore with an approximate FOV of 7 mm and 1 µm resolution. Taking a look at the commercial setups on the market, one realises that there are currently four devices which cover distinct flavours of light sheet microscopy: Lightsheet Z.1 from Carl Zeiss Microscope, Ultramicroscope from LaVision BioTec, Leica TCS SP8 DLS and iSPIM/diSPIM from Applied Scientific Instrumentation. While these systems cover very broad range of applications, they cannot be easily modified to cover different needs or additional imaging and photo-manipulation techniques. For this reason it is preferable to build a new microscope completely adapted to the sample. The microscope conceived in this project is a first prototype of the final device; once it is fully working, some upgrades might be added.

3.2- DESIGN

Considering all the required specifications (large cleared sample and large FOV), the most suitable implementation of the microscope is a mixture between an Ultramicroscope and a SPIM device. The microscope will have a horizontal orientation and will include a double-sided illumination using two cylindrical lenses, a chamber containing the sample and immersion medium, motorized movement in the Z direction and a manually positioning system in the X and Y directions (Fig. 18). Cylindrical lenses are used in this first prototype for simplicity.

The first step is choosing the most suitable laser source for my application. Venus fluorescent protein has an excitation maximum at about 515 nm and an emission maximum at about 528 nm (Fig. 15), therefore a laser of this excitation wavelength must be used. After an exhaustive search on the market considering several similar options, the chosen laser source

---

\(^4\) Venus belongs to the family of Yellow Fluorescent Protein (YFP). YFP is a genetic mutant of GFP which is originally derived from the jellyfish *Aequorea victoria*. 
for its availability and price is the TOPTICA iBeam Smart 515-S. It emits at 515 nm, has a maximum power of 100 mW, M\(^2\) of less than 1.2 and a beam diameter at 1/e\(^2\) of 1.3 mm.

![Figure 15](image)

Figure 15. Excitation and Emission spectra of YFP. Data extracted from *Fluorescence SpectraViewer* (*life technologies*).

The setup will be designed to include either the state-of-the-art camera Hamamatsu Flash 4.0, which has a sensor size in pixels of 2048 x 2048, or the Hamamatsu Orca R2 (1344 x 1024). Both cameras have a pixel size of 6.5 \(\mu\)m and as a consequence, all the calculations are equivalent. However, I will use the Flash 4.0 as a model because it is more restrictive.

Because of the peculiar structure of the LSFM, I will divide the calculations in two subsections: detection path and illumination path.

### 3.2.1 Detection Path

To make the following calculations more comprehensible, I define the coordinate system as shown in figure 18. If I wanted the FOV of the camera (FOV\(_{\text{cam}}\)) to be 7 mm with a resolution of about 1 \(\mu\)m, I would need a camera of 7000 pixels but, unfortunately, a camera like that is not currently available commercially. Therefore, the solution is to design a magnifying system with the most similar specifications to the initial requirements.

By simply multiplying the sensor size (in pixels) by the pixel size we obtain the sensor size in mm:

\[
Sensor\ size = 2048 \times 2048 \times 6.5\mu m \times \frac{1\text{mm}}{10^3\mu m} = 13.31 \times 13.31\ mm
\]

(3)

Therefore, I obtain a squared FOV\(_{\text{cam}}\) of 13.31 mm and, as expected, I need to magnify the image to obtain a FOV\(_{\text{cam}}\) of 7 mm.

\[
M = \frac{\text{sensor size}}{\text{FOV}_{\text{cam}}} = \frac{13.31\ mm}{7\ mm} = 1.9x
\]

(4)

As the FOV must be very large, I need a macro objective instead of an ordinary microscopy objective. Since the microscope is a first conception of an ulterior version, I will use a photography macro objective. I choose the NIKON Micro-NIKKOR which has 55 mm focal length and f-number 1:2.8. Photography objectives are characterized by the f-number. The f-
Design, Implementation and Test of a Routine Light Sheet Fluorescence Microscope

number $N$ (focal ratio or relative aperture) is the ratio of the lens’ focal length to the diameter of the entrance pupil:

$$N = \frac{f}{\phi} \quad (5)$$

Where $f$ is the focal length of the objective and $\phi$ is the diameter of the entrance pupil. Putting the values of the objective to the formula I obtain an entrance pupil of:

$$\phi = \frac{f}{N} = \frac{55 \text{ mm}}{2.8} = 19.6 \text{ mm} \quad (6)$$

Recalling the definition of NA given in chapter 1, doing the approximation for small angles and being in air conditions, I have that $NA \sim \theta$, and applying basic trigonometry is straightforward to see that the following relation holds:

$$\phi = 2NAf \quad (7)$$

And thus applying equation 7 I obtain the numerical aperture of the objective:

$$NA = \frac{\phi}{2f} = \frac{19.64 \text{ mm}}{2 \cdot 55 \text{ mm}} = 0.178 \quad (8)$$

With this information I proceed to calculate the resolution of my optical system applying the Rayleigh criterion. It is defined in terms of the minimum resolvable distance between two point sources of light generated from a specimen and is not dependent upon the magnification used to produce the image. According to the Rayleigh criterion, two closely spaced Airy disks are distinguishable if they are farther apart than the distance at which the principal maximum of one Airy disk coincides with the first minimum of the second Airy disk (Fig. 16). If the point sources are of equal wavelength, their Airy disks have the same diameter, and the Rayleigh criterion says that the resolution is equal to the radius of one Airy disk, measured from its point of maximum intensity to the first ring of minimum intensity.

$$r = \frac{0.61\lambda}{NA} \quad (9)$$

However, I will consider the resolution of my system to be the value of the Airy disk at the Full Width Half Maximum (FWHM) because it is more convenient when comparing experimental values to theory and it is an equally valid approximation.

$$FWHM = \frac{0.514\lambda}{NA} \quad (10)$$

\[5\] Diffraction pattern generated by a point source or by a small circular aperture.
\[6\] The derivation can be found in Additional Material.
Hence, the maximum lateral (XY) resolution one can obtain having an infinitesimal pixel with this objective is:

\[ FWHM_{X,Y} = \frac{0.514 \lambda}{NA} = \frac{0.514 \cdot 0.528 \mu m}{0.178} = 1.52 \mu m \]  

(11)

This value would be good for the resolution of the microscope if the pixel size of the camera was not limiting it. Taking the magnification previously calculated I obtain that the pixel size on the sample is:

\[ \text{px size sample} = \frac{\text{px size camera}}{M} = \frac{6.5 \mu m}{1.9x} = 3.42 \mu m/px \]  

(12)

And due to the Nyquist sampling criterion, the maximum resolving power of the system would be 6.84 \( \mu \)m, too large. To solve this problem, I redo the calculations but this time starting from the end, the required resolution.

To make use of the maximum resolving power of the system I would need the pixel size on the sample to be at least half of the resolution, 0.76 \( \mu \)m/px. And this value gives a magnification of:

\[ M = \frac{\text{px size camera}}{\text{px size sample}} = \frac{6.5 \mu m}{0.76 \mu m} = 8.55x \]  

(13)

And now the \( FOV_{\text{cam}} \) becomes:

\[ FOV_{\text{cam}} = \frac{13.31 \text{ mm}}{8.55x} = 1.56 \text{ mm} \]  

(14)

<table>
<thead>
<tr>
<th>( M )</th>
<th>( FOV_{\text{cam}} ) (mm)</th>
<th>Resolution (( \mu )m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9x</td>
<td>7</td>
<td>6.84</td>
</tr>
<tr>
<td>8.6x</td>
<td>1.56</td>
<td>1.52</td>
</tr>
</tbody>
</table>

Table 2. Summary of the results of the two calculations starting from the opposite ends.

It becomes clear in table 2 that it is impossible to design a system which fulfils both \( FOV_{\text{cam}} \) and resolution requirements simultaneously. If I obtain high resolution, the \( FOV_{\text{cam}} \) is reduced and vice-versa.
As seen in chapter 1, the magnification $M$ of a compound microscope is the relation between the focal lengths of the tube lens and the objective, so, by simply changing the tube lens I will be able to change the optical magnification. Considering that, I will design a zoom mechanism to switch between two tube lenses to obtain both the proper FOV$_{\text{cam}}$ and resolution.

To obtain 1.9x and 8.6x magnifications I need tube lenses of focal lengths:

$$f_{\text{TL}}(M = 1.9x) = M f_{\text{obj}} = 1.9 \cdot 55 \text{ mm} = 105 \text{ mm} \quad (15)$$

$$f_{\text{TL}}(M = 8.6x) = M f_{\text{obj}} = 8.6 \cdot 55 \text{ mm} = 470 \text{ mm} \quad (16)$$

After searching what tube lenses are available and redoing the calculations again I obtain the final specifications, which are detailed in table 3. The first configuration allows a full FOV$_{\text{cam}}$ of 7.32 mm and lateral resolution limited by the camera of 7.14 µm. And the zoom-in configuration makes almost full use of the optical power of the system achieving a resolution of 1.79 µm at the expense of the FOV$_{\text{cam}}$, which is reduced to 1.83 mm.

<table>
<thead>
<tr>
<th>$f_{\text{obj}}$ (mm)</th>
<th>$f_{\text{TL}}$ (mm)</th>
<th>$M$</th>
<th>FOV$_{\text{cam}}$ (mm)</th>
<th>FWHM$_{XY}$ (µm)</th>
<th>px size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>100</td>
<td>1.82x</td>
<td>7.32</td>
<td>7.15</td>
<td>3.57</td>
</tr>
<tr>
<td>55</td>
<td>400</td>
<td>7.27x</td>
<td>1.83</td>
<td>1.79</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table 3. Final specifications of the system.

There are many possible sizes of lenses and one has to choose them to be big enough to avoid vignetting$^7$. Applying basic trigonometry on figure 17 one can derive a relation between the focal lengths of the objective and tube lens, sample size and diameter of the tube lens.

Let $\alpha$ be the angular FOV of the objective and $L$ be the size of the portion of the sample to be imaged, then:

\[ \frac{h}{f_{\text{TL}}} = \frac{f_{\text{obj}}}{\alpha} \]

---

$^7$ In microscopy, when the collection lens is smaller than the size of light coming from the sample, the final image may be cropped at the edges, this phenomenon is called vignetting.
\[ \tan \alpha = \frac{L/2}{f_{obj}} \quad (17) \]

At the left hand side of the objective (Fig. 17), the angle \( \alpha \) is the same as at the right and the following relation holds:

\[ \tan \alpha = \frac{h}{f_{TL} + f_{obj}} \quad (18) \]

Combining both expressions:

\[ \frac{L}{2f_{obj}} = \frac{h}{f_{TL} + f_{obj}} \quad (19) \]

Rearranging terms I obtain the equation:

\[ h = \frac{L(f_{TL} + f_{obj})}{2f_{obj}} \quad (20) \]

Moreover, the pupil size must be considered and added to the equation:

\[ h = \frac{L(f_{TL} + f_{obj})}{2f_{obj}} + \frac{\phi_{obj}}{2} \quad (21) \]

Finally, I calculate the required diameter of the tube lenses:

\[ d_1 = 2h_1 = \frac{7.32 \cdot (100 + 55)}{55} + 19.6 = 40.2 \text{ mm} \quad (22) \]

\[ d_2 = 2h_2 = \frac{1.83 \cdot (400 + 55)}{55} + 19.6 = 34.7 \text{ mm} \quad (23) \]

### 3.2.2- Illumination Path

In this section I will discuss and calculate the dimensions of the light sheet and the different elements of the illumination path. Before entering into detail, it is mandatory to fully understand figure 18. The field of view that until now I have called FOV\(_{\text{cam}}\), corresponds to the maximum FOV that the camera is capable of imaging through the detection optics. However, in LSFM only a single plane is illuminated and, hence, it is the light sheet which determines the actual FOV. When light passes through the cylindrical lens, it focuses in one dimension (in this case \( Z \)) leaving the other (\( Y \)) unchanged and then it spreads again. As light is an infinite distribution of energy, there is not such a thing as ‘length’ or ‘thickness’ of the light sheet. What it is in fact called ‘length’, is the region where the light is sufficiently focused. Similarly, its thickness is another subjective property that can be estimated in multiple manners. Therefore, the FOV in the direction of propagation (FOV\(_X\)) is the region where light sheet is considered to be thin enough. In contrast, FOV in the perpendicular direction (FOV\(_Y\)) is simply
determined by the height of the beam passing through the lens. Hence, the FOV in both directions is directly or indirectly dictated by the entrance pupil $\phi_x \times \phi_V$ of the cylindrical lens, which determines its NA.

As the reader will notice, estimating the dimensions of the light sheet is not an easy task. Some authors have used Fraunhofer’s diffraction theory [Pawley, 2006], others have employed Gaussian beam propagation [Dodt et al., 2007] and even some have applied Heisenberg’s uncertainty principle of quantum mechanics [Engelbrecht et al., 2006]. Here, as a first approximation I will use Fraunhofer’s diffraction theory.

It is well known that the intensity of the Fraunhofer diffraction pattern of a circular aperture in the YZ plane is given by the Airy disk [McCutchen, 1963]:

$$I(z) \propto \left( \frac{J_1(z)}{z} \right)^2$$ \hspace{1cm} (24)

I will estimate the thickness of the light sheet as the diameter at which the beam intensity has fallen to 50 percent of its peak (FWHM), which is given by the same approximation as equation 10:

$$FWHM_x = \frac{0.514 \lambda}{NA}$$ \hspace{1cm} (25)

Equivalently, in the axial direction (X) the diffraction pattern takes the form of a sinc function [McCutchen, 1963]:

$$I(x) \propto \left( \frac{\sin(x)}{x} \right)^2$$ \hspace{1cm} (26)
For estimating the FOV$_X$ I will use a criterion based on the Rayleigh range. The Rayleigh range is the distance from the waist of the beam to the place where the area of its cross section is doubled, or equivalently, the place where the intensity has dropped to half its maximum value. I will consider that the Rayleigh range is the region where the light sheet is still acceptably thin, and therefore, I will take twice the Rayleigh range, i.e. FWHM$_X$, as the FOV$_X$. In this case the FWHM$_X$ is$^8$:

\[ FWHM_X = \frac{1.7718\lambda}{NA^2} \]  

(27)

**Figure 19.** Two plots of the intensity profile of diffraction patterns in the YZ plane (left) and in the X direction (right). $I(z) \propto (J_1(z)/z)^2$ and $I(x) \propto (\sin(x)/x)^2$ respectively.

Note that for my calculations I always take $n = 1$ because all lenses are placed in air.

Once the mathematical tools are ready, I proceed to calculate the properties of the elements of my illumination system. The first things to determine are the NA and focal length of the cylindrical lens.

Applying equation 27 to the low magnification mode, I obtain:

\[ NA_{cyl_1} = \sqrt{\frac{1.772\lambda}{FWHM_x}} = \sqrt{\frac{1.772\lambda}{FOV_x}} = \sqrt{\frac{1.772 \cdot 0.515 \mu m}{7320 \mu m}} = 0.0112 \]  

(28)

Hence, with a cylindrical lens of 0.0112 NA I will obtain a FOV$_X$ of 7.32 mm. On the other hand, I can obtain the desired FOV$_Y$ by simply illuminating the lens with a beam of that size, that is $\phi_Y = FOV_Y$. I repeat the calculation for the high magnification mode and I obtain:

\[ NA_{cyl_2} = \sqrt{\frac{1.772 \cdot 0.515 \mu m}{1830 \mu m}} = 0.0223 \]  

(29)

---

$^8$ See Additional Material for derivation.
Once I have calculated the NA required for each magnification I find the ideal focal length of the cylindrical lens for both cases. Applying equation 7 to both magnifications and assuming a beam size equal to the size of FOV$_y$, I obtain:

$$f_{cyl1} = \frac{FOV_y}{2NA_{cyl1}} = \frac{7.32 \text{ mm}}{2 \cdot 0.0112} = 326 \text{ mm}$$ \hspace{1cm} (30)

$$f_{cyl2} = \frac{\phi_x}{2NA_{cyl2}} = \frac{1.83 \text{ mm}}{2 \cdot 0.0223} = 41.0 \text{ mm}$$ \hspace{1cm} (31)

Both values are very dissimilar from each other so, the goal now is to find a trade-off between the two options in order to optimize the system.

In table 4 I have set a fixed value for the focal length of the cylindrical lens and I calculate the width of the illumination pupil that provides the desired FOV$_x$. It becomes evident that if I want to use the same cylindrical lens for both magnifications I need to change the illumination pupil. To achieve it I will use a variable rectangular slit which has a maximum aperture of 12 x 12 mm. Consequently, the variable slit will allow changing the area of illumination on the cylindrical lens and thus its NA. Therefore the dimensions of the light sheet can be modified by simply opening or closing the slit.

<table>
<thead>
<tr>
<th>$f_{cyl}$ (mm)</th>
<th>$\phi_x$ (mm)</th>
<th>$\phi_x$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.12</td>
<td>2.23</td>
</tr>
<tr>
<td>100</td>
<td>2.23</td>
<td>4.47</td>
</tr>
<tr>
<td>150</td>
<td>3.35</td>
<td>6.70</td>
</tr>
<tr>
<td>200</td>
<td>4.47</td>
<td>8.93</td>
</tr>
<tr>
<td>250</td>
<td>5.58</td>
<td>11.16</td>
</tr>
<tr>
<td>300</td>
<td>6.70</td>
<td>13.38</td>
</tr>
</tbody>
</table>

Table 4. Width of the entrance pupil of the cylindrical lens as a function of its focal length for both magnifications.

However, before choosing the lens, another consideration should be taken into account. In this setup the sample can be immersed in water (or any other medium) but the objective is designed to work in air conditions. This difference in the index of refraction indicates that the position of the focal plane will change axially (Z direction) as a function of the length of the propagated beam inside the medium, resulting in an out-of-focus region in the image. To obtain a good LSFM image, the light sheet must always coincide exactly with the focal plane of the objective and, therefore, one solution to this problem is to move the light sheet in the Z direction in order to compensate the effect of water and keep always the light sheet and focal plane coinciding. The importance of this effect is quantified below.
Let \( f \) be the focal length of the objective and \( \phi \) its pupil, let \( l \) be the distance between the objective and the chamber, let \( d \) be the distance from the edge of the chamber to the focal point. Now, I apply Snell’s Law and trigonometry:

\[
n \sin \alpha = n' \sin \alpha' \quad (32)
\]

\[
\alpha' = \arcsin \left( \frac{n}{n'} \sin \alpha \right) \quad (33)
\]

\[
\frac{h}{d} = \tan \alpha \quad (34)
\]

\[
\frac{h}{d'} = \tan \alpha' \quad (35)
\]

\[
\frac{d'}{d} = \frac{\tan \alpha}{\tan \alpha'} \quad (36)
\]

\[
\tan \alpha = \frac{\phi/2}{f} = NA \quad (37)
\]

Since the medium used in all my experiments will be water, I take \( n = 1 \) and \( n' = 1.333 \) and obtain:

\[
\alpha = \arctan NA = \arctan 0.1785 = 0.1767 \quad (38)
\]

\[
\alpha' = \arcsin \left( \frac{3}{4} \sin 0.1767 \right) = 0.1322 \quad (39)
\]

\[
\frac{d'}{d} = \frac{\tan \alpha}{\tan \alpha'} = \frac{0.1785}{0.1330} = 1.343 \quad (40)
\]
Finally I obtain the relation:

\[ \Delta d' = 1.343 \Delta d \]  \hspace{1cm} (41)

The result says that the effect of water is by no means negligible and a correction is needed. To achieve it, I will need to place a mirror on a translation stage between the cylindrical lens and the chamber to keep the image always in focus. This becomes a big mechanical constraint when choosing the cylindrical lens because I will have to leave enough space to allow the steering of the different elements.

Figures 21 A, B and C, show a comparison of the FOV and light sheet thickness between some combinations of cylindrical lenses and pupils. The FOV requirements can be exactly fulfilled with any lens with a focal length comprised between 50 mm and 250 mm. The 300 mm lens is discarded because its performance is limited by the maximum width of the slit (Table 4). Among the other available lenses, the most suitable for my application is the 250 mm because not only is it capable of achieving the desired FOV, but also its long focal length gives more mechanical flexibility to build the microscope. The final specifications of the system using the 250 mm cylindrical lens are presented in table 5. A quick calculation of the axial resolution for an equivalent widefield microscope with the same detection optics as the Macro SPIM confirms almost a threefold improvement by the implemented microscope.

\[ FWHM_z = \frac{1.7718\lambda}{NA^2} = \frac{1.7718 \cdot 0.528 \, \mu m}{0.178^2} = 29.53 \, \mu m \]  \hspace{1cm} (42)

<table>
<thead>
<tr>
<th>M</th>
<th>FOV (mm)</th>
<th>Thickness (\mu m)</th>
<th>( \phi_x ) (mm)</th>
<th>( \phi_y ) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.82x</td>
<td>7.32 x 7.32</td>
<td>23.72</td>
<td>5.58</td>
<td>7.32</td>
</tr>
<tr>
<td>7.27x</td>
<td>1.83 x 1.83</td>
<td>11.80</td>
<td>11.16</td>
<td>1.83</td>
</tr>
</tbody>
</table>

Table 5. Final dimensions of the light sheet and illumination pupil of the cylindrical lens.

Furthermore, by simply adjusting the slit width, the uniformity and the thickness of the light sheet can be modified. For any cylindrical lens, when the slit widens, the light sheet becomes thinner and the FOV smaller. For example, in the low magnification mode the light sheet thickness can reduced to just 20 \( \mu m \) by setting the pupil to be 6.5 mm instead of 5.6 mm.

Finally, to fill the entire slit I need to expand the laser from 1.3 mm of diameter to at least 12 mm. I will use a Keplerian telescope using a 16.6 mm objective and a 200 mm lens obtaining a magnification of 12.05x which expands the laser to 15.7 mm.
**Figure 21.** Comparison of FOV_x and light sheet thickness between different configurations. The FOV_x and light sheet thickness have been estimated by equations 25 and 27 respectively. Distance between zeroes is displayed only to illustrate how different the estimation criteria can be. **A** and **C** configurations fulfil FOV_x requirements (200 mm and 250 mm lenses respectively). **B**: Using the 300 mm lens it is impossible to achieve the desired FOV_x. **C** and **D** correspond to the two magnification modes of the system using the chosen 250 mm cylinder lens.
3.3- BUILDING AND PRE-ALIGMENT

The next steps of the microscope implementation are gathering the required material, building it in the lab and aligning the whole setup with precision. Figure 22 shows a schematic design of the system on the optical table and the actual system is shown in figure 23. In the following paragraphs I give some more details of the important parts of the microscope and the pre-alignment process.

3.3.1- Lasers

The system contains two lasers, a 515 nm diode laser which has a maximum output power of 100 mW, and a 633 nm He-Ne laser with 5 mW of power (Fig. 24). The first one is the excitation laser which generates the fluorescence of the sample and its fully controlled digitally. The second one is used to pre-align the setup and to operate the microscope in scattering mode. In front of the laser diode there is an attenuator to obtain a better control of its power, and in front of the He-Ne one there is a filter wheel also to attenuate it. A flip mirror allows switching between the two lasers.
3.3.2- **Light Sheet generation**

Once the beam has been expanded, it passes through the rectangular slit and a flip mirror determines which one of the two illumination arms the light goes to. Then the cylindrical lens focuses the light in one dimension and finally a mirror directs the light sheet to the sample chamber. The cylindrical lens is mounted on a translation stage in order to position the beam waist of the light sheet exactly at the centre of the FOV. The mount of the cylindrical lens rotates and allows setting the light sheet completely vertical (Fig. 25). The last mirror is also placed on a translation stage to correct the effect of the immersion medium as explained in section 3.2.2.

![Figure 25. Photograph of a cylindrical lens installed on a rotating mount.](image)

3.3.3- **Chamber**

The chamber is a 2 x 2 x 2 cm container which is filled with the immersion medium of the sample if required (Fig. 27). It stands on two translation stages (Fig. 26) that allow a precise manual positioning of the sample in the X and Y directions. And finally, the whole structure is mounted on a motor that moves it along the Z direction (Fig. 27).

![Figure 26. Photograph of the final part of the illumination system. At the top corner in the background, the detection system is also partially visible.](image)

![Figure 27. Detail of the chamber and detection objective.](image)
3.3.4- Detection System

Just after the detection objective (Fig. 28), a longpass filter blocks the scattered laser light and only allows fluorescently emitted light to pass through it. It reduces enormously the noise in the images. Achromatic doublets are used as tube lenses because they limit chromatic and spherical aberrations. Both doublets are mounted on fixed supports and a translation stage moves two mirrors that enable the user to switch between the two magnification modes.

3.3.5- Diaphragms and Pinholes

Diaphragms and pinholes are placed at strategic points of the setup and their function is to give a reference to align the laser. Some of them are provisional and are used just during the pre-alignment process, others are fixed and act as a reference if one wants to add a new element to the system. To align the elements of each path of the setup, one has to place two pinholes or diaphragms as separate as possible of each other and using two mirrors has to make the laser pass through them. Once the laser goes perfectly straight, lenses and other elements are positioned according to the imaginary line drawn by the reference laser.

3.4- LIGHT SHEET CHARACTERIZATION AND FINE ALIGNMENT

Before imaging any sample, the microscope must be precisely aligned. Due to the unique design of a light sheet microscope, its alignment process differs a lot from traditional fluorescence microscopy techniques. Here, the light sheet needs to be aligned with respect to the focal plane of the detection arm. One has to ensure that the light sheet is exactly at the focal plane of the objective with its waist at the centre of the FOV and perfectly orthogonal to the detection axis. The procedure of fine alignment that I follow can be divided in two stages: mirror at 45º and fluorescent medium.

3.4.1- Mirror at 45º

This first method allows me to do a first alignment of the light sheet and at the same time makes it possible to characterize its actual thickness and uniformity to compare them to the theoretical results.

A reflective mirror is placed in the focal plane of the detection arm and tilted by 45º into the direction of the light sheet (Fig. 31). By moving the mirror along the illumination axis (X), one...
can see the light sheet width at different positions and align it to be centred. In Fig. 30 A, C and E, I have taken an image of the light sheet at different positions and represented them in a single image. The result is a set of vertical lines that correspond to the light sheet width at different positions along the X axis. This way, I can see its uniformity and determine if the Rayleigh range is large enough. In B, D and F, the intensity profile of the light sheet at each X position are superimposed and the thickness and uniformity along the axis can be easily seen. The first pair of images (A and B) correspond to the low magnification mode with a slit width of 6.5 mm. Even though it is not the optimal configuration, the light sheet is clearly uniform and just diverges a little bit at the edges of the image. In addition, its width at the waist matches the theoretical value for this slit size. The second pair of images (C and D) correspond also to the low magnification mode but with a slit width of 12 mm. This time, the light sheet diverges enormously and also reduces its thickness at the waist as expected. Finally, the last pair of images (E and F) correspond to the 12 mm width slit in the high magnification mode. Here the light sheet remains uniform along the entire FOV and its thickness is the same as in the previous pair (note that image E would correspond to the magnified central region of C). Also note that the characterization data shown is figure 30 has been obtained using the 515 nm laser, whereas figure 31 was taken with the 633 nm laser ON.

Figure 30. A, C and E: Images of the light sheet at different positions of the illumination axis (X). B, D and F: Superimposition of the light sheet profiles at the X positions labelled with colour lines in the left images. In A and C the distance between lines is 400 µm and in E 100 µm.
3.4.2- Fluorescent Medium

With the previous method, the light sheet can be precisely aligned and its dimensions determined. However, it has a major drawback: the mirror is not immersed in water. As discussed previously, a mismatch in the index of refraction results in a shift of the focal plane position. Therefore, the position of the cylindrical lens has to be adjusted to make the light sheet waist coincide with the centre of the FOV. In order to do that, I fill the chamber with a fluorescent solution of water and fluorescein\textsuperscript{9}. This fluorescent medium makes the light sheet visible with a naked eye and at the display.

Fig. 32 A shows a picture of the chamber filled with the fluorescein solution and the light sheet rotated 90° with respect to its typical position. By moving the cylindrical lens on a translation stage, I obtain image B which shows that the light sheet waist is perfectly centred in the FOV. Note that the light sheet has also to be moved in the Z direction to match the detection objective’s focal plane but this will be adjusted later for each specific sample.

In addition, the fluorescent solution also makes it possible to see whether the vertical (Y) diffraction effects are important or not. Figs. 32 C-H show the effects of diffraction when the slit height is reduced. When the slit is almost closed ($\phi_Y$ much less than 1 mm), the light sheet has the shape of the diffraction pattern of a slit aperture, i.e. a sinc function. As the height of the slit is increased, the diffraction pattern changes and more fringes appear. When the slit reaches a height of about 10 mm, the distribution of light becomes practically homogenous. In conclusion, diffraction sets a lower limit for the height of the slit. Below this limit, fringes appear in the light sheet and the illumination of the sample is not uniform, resulting in dark regions in the image. The patterns obtained are completely in accordance with the results obtained by Harris et al. [Harris et al., 1969].

\textsuperscript{9} Fluorescein is a synthetic organic compound (C\textsubscript{20}H\textsubscript{12}O\textsubscript{5}) soluble in water which has very intense fluorescence.
3.5- CONTROL SOFTWARE

The control software\textsuperscript{10} has been implemented in LabVIEW 2011 SP1 starting from scratch and ending up in a fully functional, scalable and robust program capable of controlling the Macro-SPIM microscope. Its main features are the possibility of seeing the sample in real time using the Live mode, capturing images at any time by just pressing a button (Snap) and the most important, Stack. In this mode the motor is set to move from one end of the sample to the other in stepwise motion. At each step the camera captures an image and this way a 3D image is obtained. All images are saved in the widely used TIFF (Tagged Image File Format) format, snaps are saved as individual images and stacks in a single TIFF file with multiple pages.

The user interface has been accurately designed to be easy to use for non-specialist users (Fig. 33). At the upper right corner there is a selector where the user can choose the camera, objective and tube lens and obtain the magnification, FOV and pixel size. Below that, there are the Live, Snap, Stack and Quit buttons. In the middle, the user can find the camera settings and their maximum and minimum values as well as their sensitivity. Two buttons move the stage to the left or to the right and next to them the motor settings can be set. At the bottom there are the stack parameters which are used to determine the span of the motor and the step size. There are also two indicators that show the total number of images the stack will have and the current one. Finally, a slide shows the percentage of completion of the stack acquisition. On the left

\textsuperscript{10} Part of the original code is displayed in Additional Material.
hand side the display shows an image of a *C. elegans* worm. Images can be displayed in four different colour look-up tables: Hot Red, Spectrum, Fire and Black and White.

![User Interface of the control program.](image)

**Figure 33.** User Interface of the control program.

The program is based on an event driven state machine. A state machine is a programming architecture that can be used to implement any algorithm that can be explicitly described by a state diagram or flowchart. It introduces a way to create a program such that it responds to user events (for example keystrokes and mouse events). A state machine consists of a set of states and a case selector that determines which state the program transitions to next. In LabVIEW, a state machine is created with a while loop, a shift register, a case structure, and some form of case selector. The while loop is the main program loop, it executes one case per iteration and runs until the Quit button is pressed. The shift register keeps track of which case should be the next to execute (which is decided by the case selector code). Finally, each case of the case structure contains the action for one specific user action.

The program begins by initializing the camera and the motor, setting the default values for all their settings. Once the hardware is ready, the program enters in the Idle case which contains an event structure. The event structure waits until an event occurs, then executes the appropriate event case to handle that event. In this program the event structure contains 17 events which can be grouped in 9 blocks: Settings, Define Home, Go Home, Live, Snap, Velocity, Stack, Step and Quit. Once the event has been selected the program goes to the specific case of the state machine and the action is performed. The main actions of each event are summarized in the following lines. Moreover, a dataflow diagram is displayed to make the program more comprehensible (Fig. 34).
3.5.1- **Timeout**

This event is activated after 1 ms of inactivity and it is not connected to any other case of the state machine. It either waits for an action or takes images continually when the Live button is ON. Moreover, at each iteration of the while loop it always provides the current position of the stage.

3.5.2- **Settings**

Under this name there are grouped many different settings of the camera and the stack parameters: Exposure Time, Contrast Gain, Contrast Offset, Binning, Bits and Stack Parameters. When the user modifies any of these values, the program runs a process that applies the changes.

3.5.3- **Define Home**

Sets the current position as Home, i.e., the current position becomes the origin ($z = 0$).

3.5.4- **Go Home**

The motor moves the stage to the Home position.

3.5.5- **Live**

When the user presses the Live button, the event case drives the program to the Play/Stop case. First, the camera is set to work in sequence mode and then, the program goes to Idle and stays at the Timeout event in which the camera acquires an image at each iteration. Note that in this mode images are not saved. When the Live button is deactivated, the Play/Stop case stops the camera and the program stays at Timeout doing nothing.

3.5.6- **Snap**

As the name indicates, this event takes and saves a single image of the sample.

3.5.7- **Velocity**

This event sets the velocity at which the motor moves, it has three possible values: Fine, Medium and Coarse.

3.5.8- **Stack**

This is perhaps the most complicated process of the software since it involves the coordinated control of the motor and the camera to obtain a stack of images. The process begins when the user presses the Stack button after setting the starting and ending points along the $Z$ axis as well as the step size of the motor. At that moment, the program checks if the Live mode is ON and if it is true, the program is directed to Play/Stop where it is then switched off. After that, the Stack case checks whether the current iteration is the first or not. If the iteration is the first one, a new TIFF file is created. Moreover, if the stage is not at the starting position the motor is moved to $Z_{\text{min}}$ and the position is displayed on the screen. After moving the stage, the
camera is prepared in Play/Stop case and the first image is acquired in the Get Frame case. Then, one iteration is added to the counter and the program checks if more steps are required. While the answer is true, the program keeps running and takes an image at every step until the motor has scanned the whole sample. It is then when the camera is stopped, the TIFF file closed, and the program finally returns to the Timeout event case.

3.5.9- Step

In this event the user can set the step size and choose to move the motor to the right (negative Z) or to the left (positive Z).

3.5.10- Quit

When the program needs to be stopped, the user just needs to press the Quit button and it will automatically stop the motor and the camera.

Figure 34. Flowchart diagram of the software control. Green rectangles with rounded corners represent the processes required to start or end the program. Green rectangles represent the actions or processes that take place in each case of the state machine. Yellow rectangles stand for some of the most relevant subprocesses. White trapeziums represent an event or group of events inside the Idle case. Blue diamonds represent decisions.
3.6- SAMPLE PREPARATION

All samples I prepare for my experiments (C. elegans and beads) are embedded in low melting agarose blocks of variable concentrations. The agarose blocks are prepared by melting agarose in a microwave at 50°C and pouring approximately 1.25 mL of it in a 1.5 mL centrifuge tube (Eppendorf). While it is still liquid, the specimen is inserted and mixed using a vortex spinning machine. It is then placed in a fridge at about 5°C for a few minutes until it solidifies. Finally, the agarose block is gently extracted from the Eppendorf with a small spatula and deposited inside the chamber, which is filled with water.

3.6.1- Scattering beads

The first tests are done with Copolymer Microsphere Suspensions (Thermo Scientific) of a mean diameter of 55 µm. Copolymer microspheres are composed of polystyrene polymer, cross-linked with divinylbenzene (DVB). Microspheres are deposited inside 1.5-2% low melting agarose at a dilution of about 0.1%.

3.6.2- Fluorescent beads

For characterizing the resolution of my system I use 0.930 ± 0.01 µm Y Estapor Fluorescent Microspheres. They are much smaller than the resolution of the low magnification mode and, therefore, when imaging them I will obtain the PSF of the system. However, for the high magnification mode I will be close to Nyquist limit but it will probably be enough as well. Fluorescent beads are simply diluted in water and then mixed with 1.5-2% low melting agarose obtaining a final dilution of 1:1000000.

3.6.3- C. elegans

To test the system for biological applications, samples containing Caenorhabditis elegans (C. elegans) nematodes were prepared (Fig. 35). C. elegans is a small nematode (roundworm) that lives in many parts of the world and survives by feeding on microbes, primarily bacteria. It is an important model system for biological research in many fields including genomics, cell biology, neuroscience and aging. Some of its many advantages are its short life cycle, compact genome and small size. The adult body is anatomically simple with about 1000 somatic cells and 302 neurons. The animal can be maintained in the laboratory where it is grown on agar plates with E. coli as the food source. There are

![Figure 35 Artist’s conception of a C. elegans nematode.](image)
two *C. elegans* sexes: a self-fertilizing hermaphrodite (XX) and a male (XO). Males arise infrequently, about 1 male per 1000 born worms.

For my experiments I will use genetically modified worms which have the pharynx and neurons labelled with yellow fluorescent protein (YFP). To ensure that their fluorophores can be well excited with the 515 nm laser, I measure the emission spectrum of a worm using a spectral confocal microscope (Fig. 36). The result shows that the fluorophore is YFP indeed and that the pharynx and neurons can be excited perfectly at 515 nm.

![Figure 36 Emission spectrum of the genetically modified C. elegans.](image)

Sample preparation of worms is a more laborious than beads. First, I put a drop of anaesthesia (45 mM sodium azide, NaN₃) on a glass slide. Secondly, I pick up between 10 and 30 adult worms using a small picker, employing a stereomicroscope to see them. After waiting about 15-20 minutes for the worms to become anaesthetised, I put them into the melted 1-1.5% agarose using a pipette. To ensure they do not wake up during the experiments, I also added 100 µL of anaesthesia to the agarose.
4- **EXPERIMENTAL RESULTS**

Once the system has been built and precisely aligned, I proceed to test its performance doing various tests using different samples and imaging modalities. For my experiments I use the camera Hamamatsu Orca R2 which has a smaller FOV than the Flash 4.0.

4.1- **BRIGHT FIELD**

For the first preliminary tests I create a simple bright field illumination using a flexible LED lamp and a diffusor to image a resolution test pattern and *C. elegans* worms. This first evaluation will provide me information of just the detection system.

4.1.1- **Standard USAF 1951 Resolution Test Pattern**

To test the resolution performance of the microscope in bright field, I use the Standard USAF 1951 Resolution Test Pattern. The test target consists of sets of three lines separated by spaces equal to the line width. The inverse of the line frequency gives the resolution in mm. The resolution limit of a system is the last group of bars that can be seen and correctly counted in both horizontal and vertical directions. In this target, resolution values lie within 4 mm to 4.42 μm. This test target not only enables me to determine the resolution limit, but also to calculate the pixel size and FOV experimentally and compare them to their theoretical values.

First, I do the analysis for the 1.82x mode. Zooming in a well resolved region I measure the periodicity of the lines in pixels giving me a value of 9 px. Since the line frequency of this region is 32 lines/mm, the length of a period is 0.031 mm (Fig. 37). As one period contains 9 pixels I can calculate the pixel size by doing a simple division obtaining an approximate value of 3.5 μm/px. Making the product between the pixel size and the number of pixels of the camera I obtain a FOV of 4704 x 3587 μm.

Finally I zoom in the smallest region of the target (Fig. 38 C) and I find that the smallest well resolved set of lines is the 143mm⁻¹ which corresponds to a resolution limit of about 6.99 μm. It is the best resolution one can obtain in this configuration because it is just at the limit of Nyquist sampling criterion, two pixels per detail of the object.

Doing the same calculations for the 7.27x magnification I obtain similar results. The resolution of this configuration is too small for this test pattern and I can just set an upper limit of it. In both cases all measured values diverge less than 2% from the theoretical ones and, therefore, I can confirm that the detection system is well designed and properly aligned (Table 6).
Images obtained using both magnifications show an overall good performance of the detection optics of the microscope because it is capable of displaying some internal details of the worm. During the experiment, worms woke up and moved along the image, leaving a trail behind, which can be seen in Fig. 40 B. Worms do not appear completely in focus because they are three-dimensionally oriented and it is impossible to obtain a single entirely

<table>
<thead>
<tr>
<th></th>
<th>1.82x</th>
<th>7.27x</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Theoretical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pixel Size (µm)</td>
<td>3.57</td>
<td>0.89</td>
</tr>
<tr>
<td>FOV (µm)</td>
<td>4762 x 3655</td>
<td>1202 x 911</td>
</tr>
<tr>
<td><strong>Experimental</strong></td>
<td>3.5</td>
<td>0.87</td>
</tr>
<tr>
<td>Resolution (µm)</td>
<td>7.14</td>
<td>1.79</td>
</tr>
</tbody>
</table>

**Table 6.** Summary of the results of the performance test compared to their theoretical values.

**Figure 38.** USAF 1951 Resolution Test Pattern. A and B correspond to full FOV images at 1.82x and 7.27x magnifications respectively. C and D are zoomed in regions of them.

**4.1.2- C. elegans**

Images obtained using both magnifications show an overall good performance of the detection optics of the microscope because it is capable of displaying some internal details of the worm. During the experiment, worms woke up and moved along the image, leaving a trail behind, which can be seen in Fig. 40 B. Worms do not appear completely in focus because they are three-dimensionally oriented and it is impossible to obtain a single entirely

**Figure 39.** Bright field image of a C. elegans acquired in the 7.27x magnification mode.
focused image. Furthermore, the bright field image shows all the dirtiness present in the agarose block deteriorating the image quality.

![Figure 40. A: Bright field image of a C. elegans acquired in the 1.82x magnification mode. B: Zoomed in detail of A.](image)

### 4.2- SCATTERING

Until now I have just analysed the performance of the detection system. Here I present the preliminary tests of the whole microscope. While the 515 nm laser is not available, I use the 633 nm He-Ne laser to realize some scattering tests.

To begin with, I take a sample of scattering beads and I illuminate it with the light sheet. As the microspheres are transparent, only the points where the light enters and leaves the sphere are visible in the image (Fig. 41 A and B). If one takes a closer look, will even see its shiny contour. From the image one can infer that the size of the particle is approximately 55.6 µm. A picture of the sample from above (Fig 41 C) clearly shows how the light sheet spreads as it traverses the sample. This strengthens the supposition that for large scattering samples, dual sided illumination is required. Finally, I test the microscope with a C. elegans worm (Fig 41 D) obtaining a good image of its silhouette.

![Figure 41. A and B: Microspheres in the 7.27x and 1.82x modes respectively. A yellow circle indicates the shape of the bead. C: Top view of the microspheres sample illuminated with the light sheet. D: C. elegans image in the low magnification mode.](image)

### 4.3- FLUORESCENCE

Finally, I test the system it its original operation mode using the 515 nm laser to image fluorescent beads and genetically modified C. elegans worms.
4.3.1- Fluorescent Beads

Small fluorescent beads are the best samples to characterize the resolution performance of a microscope. If they are sufficiently small, the microscope images them as punctual sources and, as a result, the PSF of the system is obtained. The size and shape of the PSF gives us information of the microscope’s state.

Figure 42. PSFs of the 1.82x mode. A and B: Cropped images of the XY and YZ plane respectively. C and D: Detail of a single bead in the XY and YZ plane respectively.

Figure 42 A shows a 1600 x 1000 µm portion of the FOV in the 1.82x magnification mode with a slit width of 6.5 mm. A closer look (Fig. 42 C and D) shows that the lateral resolution of the microscope in this configuration is limited by the pixel size of the camera as expected. In the axial direction (Z) of the PSF, its size is approximately 19.83 µm. Both values match almost perfectly with the theoretical estimations and, thus, I conclude that the resolution performance in this mode is correct.

Figure 43. PSFs of the 7.27x mode. A and B: Cropped images of the XY and YZ plane respectively. C and D: Detail of a single bead in the XY and YZ plane respectively.

Figures 43 A and B show a piece of the XY and YZ planes respectively. In this case, pixels are smaller and the resolution is limited by diffraction. However, the microscope does not achieve the theoretical values of the resolution because it has some spherical aberration. Spherical aberration might come from the objective, tube lens or it can even be caused by the change in index of refraction that occurs at the chamber (water-glass-air). Further studies are required to understand and solve the problem. Nevertheless, eliminating this aberration is not crucial for my experiments. It must be pointed out that
spherical aberration might also be present in the low magnification mode, but, it cannot be seen because the resolution is smaller than the pixel size. Table 7 shows a summary of both the theoretical and experimental values of the resolution. It illustrates that the microscope has an overall satisfactory performance despite the twofold error in the lateral resolution in the high magnification mode.

<table>
<thead>
<tr>
<th></th>
<th>1.82x</th>
<th>7.27x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral Resolution (µm)</td>
<td>7.15</td>
<td>1.52</td>
</tr>
<tr>
<td>Experimental</td>
<td>7.23</td>
<td>2.91</td>
</tr>
<tr>
<td>Axial Resolution (µm)</td>
<td>20.36</td>
<td>11.03</td>
</tr>
<tr>
<td>Experimental</td>
<td>19.83</td>
<td>10.84</td>
</tr>
</tbody>
</table>

Table 7. Theoretical and experimental values of lateral and axial resolution of the two magnification modes. For the 1.82x mode the slit width is 6.5 mm and for the 7.27x mode 12 mm. The value of the axial resolution of the 7.27x mode has been taken as the FWHM of the major lobe.

4.3.2- C. elegans

In the end, after all the adjustments previously detailed, the microscope is ready to image fluorescent living biological samples. Similarly to the experiments seen in previous sections, I use C. elegans worms embedded in low melting agarose. All images of this section show either the entire worm or just a part of it. The bottle-shaped structure at one end of the worm is the pharynx, the organ responsible of transporting bacteria from the mouth to the intestine. The dorsal and ventral nerve cords run the length of the worm from head to tail and can be easily identified as two thick lines at the edges of the worm. Moreover, if one takes a closer look to the images, will see individual neurons inside the worm. As C. elegans are relatively small samples, dual sided illumination is not required to obtain well-focused images. The laser power of the light sheet at the sample is about 90 µW for all the experiments.

![Image](image.png)

Figure 44. Single slices of Macro SPIM stacks of C. elegans at 7.27x magnification. A: Full FOV image. B: Zoomed in detail of A.

One of the main advantages of this implementation of the LSFM is the possibility to switch between two magnifications. If one wants to image a small detail of the specimen, just has to select the 400 mm tube lens (Fig. 44). Otherwise, by simply switching to the 100 mm tube lens, a more general image of the sample is obtained (Fig. 45).
The next step is to measure the optical sectioning capability of the system and determine whether it has been well designed or not. First, I acquire a stack of images in full field illumination conditions, that is, I illuminate the whole sample directly with the expanded beam without the cylindrical lens. The result is an image that looks very similar to an epifluorescence one. The XY maximum projection (Fig. 46 A) shows a great amount of neurons in high detail but the worm has a blurred shadow around produced by out-of-focus light coming from other planes. This lack of optical sectioning becomes more evident in the XZ and YZ maximum projections of the 3D image (Fig. 46 B and C). These images are so blurred that it is impossible to identify any part of the worm.

On the other hand, when the specimen is illuminated with the light sheet, just a single plane emits light and the blur around the details disappears, leaving a sharper image (Figs. 44, 45, 47, 48 and 49). Doing again the orthogonal projections I obtain much better images in which the optical sectioning is evident (Fig. 47 and 49, B and C). XZ and YZ projection images are apparently worse than XY simply because the microscope’s axial resolution is lower than the lateral.
As discussed in section 3.2.2, the presence of water inside the chamber produces a mismatch between the light sheet position and the focal plane of the detection objective. After a few steps of the stack acquisition, the defocus produced by the spherical aberration is so important that the problem must be fixed. This problem is corrected by moving the light sheet in the Z direction in order to make it coincide with the focal plane. Figure 48 A and B show two Z maximum projections of the same worm which have different regions in focus. A has the pharynx in focus and B just the opposite end of the body. The distance travelled by the motor for obtaining the whole 3D stack of images is 208 µm. According to equation 41, the light sheet should be moved 279 µm, and in practice I move it 270 µm obtaining two images with the two opposite ends in focus. Finally, by stitching them I obtain a final focused image. Two small arrows in Figure 48 A and B point at the...
focused points of each image. They correspond to the pharynx and the vulva of the hermaphrodite worm, respectively.

![Image](image_url)

**Figure 48.** Stitching of two stacks of a *C. elegans* with 7.27x magnification. A and B: Maximum projections of the original stacks. C: Maximum projection of the final fused image.

Finally, I show three orthogonal projections obtained with the low magnification mode of the microscope (Fig. 49). Images in this mode exhibit almost isotropic resolution. In this figure, the reader may notice some important differences in worm’s anatomy. The specimen in the figure is a death hermaphrodite full of its own offspring. Under some conditions (age, mutations, etc.) the eggs can hatch inside of the progenitor [Mosser, T. et. al., 2011]. As a result, the recently born worms can survive for some hours eating the internal tissues of their own progenitor. Images are a little bit saturated in order to make more visible the pharynxes of the offspring.

![Image](image_url)

**Figure 49.** Orthogonal maximum projections of a Macro SPIM stack of *C. elegans* with 1.82x magnification. A: XY projection. B: XZ projection. C: YZ projection.
5- CONCLUSIONS

In this project I have successfully designed, implemented and tested a light sheet fluorescence microscope which fulfils the needs of the SLN Facility at ICFO in fast three-dimensional imaging of large biological microscopic samples. It has been tested in three different imaging modalities: bright field, scattering and fluorescence, and with different samples including scattering beads, fluorescent beads and C. elegans worms. As the conceived microscope is a first prototype, its performance limits have been analysed and determined.

It is capable of imaging a maximum field of view of 7.33 mm with a lateral resolution of up to 2.91 μm. It provides intrinsic optical sectioning achieving an axial resolution of 10.84 μm. Hence, it meets the initial requirements with accuracy except from the lateral resolution in the high magnification mode, which is reduced due to effect of spherical aberration. If in the future it needs to be further corrected, it can be achieved, for example, using adaptive optics.

It has been proved that the Macro SPIM microscope provides a threefold increase in axial resolution with respect to a widefield microscope. Despite this great improvement, the resolution is not isotropic yet. In order to achieve the same resolution in all directions, a rotation motor can be added to the setup. This motor would offer the possibility of acquiring stacks of images from different angles and then applying multiview fusion to obtain an image with isotropic resolution. Nevertheless, this change in the setup is not straightforward due to mechanical constraints. The best way of doing it would be to rotate the sample from the top instead of rotating the whole chamber. In this new approach, the sample would hang from above and the preparation protocol would have to change. As sample holding is still a hot research topic, there is not a standard way of doing it, and therefore, some different holding methods should be tried.

To reduce the photobleaching of the sample, the light sheet height should correspond to the field of view of the camera. However, it has been seen that if the height of the rectangular slit is less than 10 mm, its diffraction pattern appears, resulting in dark stripes in the image. One way to minimize this phenomenon is to place the slit just in front of each cylindrical lens and also to reduce their focal length.

As the sample is moved along the detection axis, water produces a mismatch between the position of the light sheet and the focal plane of the detection objective, resulting in a half defocused image. The problem has been initially fixed by placing a mirror on a manual translation stage between the cylindrical lens and the chamber in order to keep the light sheet always in focus. An interesting upgrade of the system would be the introduction of a motor to move that mirror in a coordinate fashion with chamber. In this way, as the sample is scanned in the Z direction the light sheet would follow the focal plane resulting in a well-focused image in the entire field of view. Motorization can also be implemented in the switching mechanism between the two tube lenses to make it possible to change magnification automatically using the control software.
Another important upgrade would be the replacement of the flip mirror which selects the illumination path by a beam splitter and two shutters. This would also enable the user to control digitally which side the sample is illuminated from, and it would even give the possibility of acquiring two stacks simultaneously. At each step of the stack, two images would be captured automatically by simply opening and closing the shutters. This would ensure that the conditions of the sample for both stacks are constant.

Finally, the last relevant upgrade would be the incorporation of a 488 nm laser to make the microscope capable of imaging samples labelled with green fluorescent protein (GFP), which is one of the most common fluorophores in biological samples.

In conclusion, the implemented Macro SPIM microscope is fully functional, user-friendly and flexible to incorporate many upgrades adapted to the specific needs of the samples. It has been tested with living biological samples and shortly it will be used to image clarified mouse brains. It opens the doors to important collaborative projects between ICFO and other institutions which require the tools to image large samples in the microscopic domain such as developing embryos or whole organs in the pursuit of understanding life processes. Once we get a dynamic picture of these processes, we will be able to understand how they actually work and hope to find solutions to diseases that are caused by their failure.
6- REFERENCES