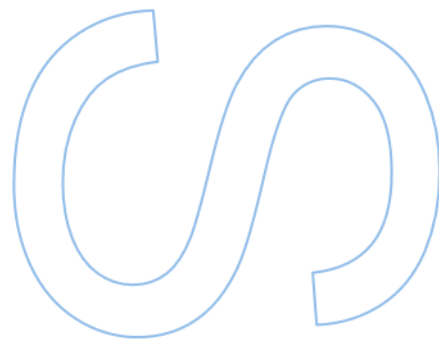
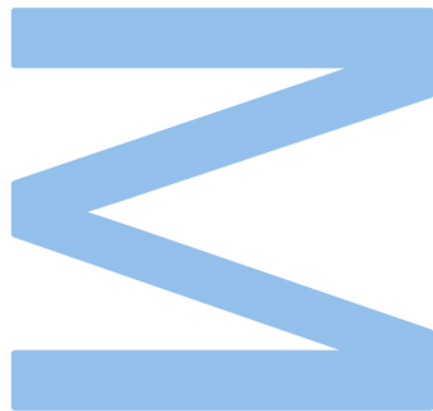


VitaSlice: software development and system optimization of a custom Light Sheet Fluorescence Microscope

Simão Peniche Seixas

Master in Engineering Physics
Department of Physics and Astronomy
Faculty of Sciences of the University of Porto
2025



VitaSlice: software development and system optimization of a custom Light Sheet Fluorescence Microscope

Simão Peniche Seixas

Internship Report carried out as part of the Master in
Engineering Physics
Department of Physics and Astronomy
2025

Supervisor

Prof. Paulo Marques, Auxiliar Professor, Faculty of Sciences of the
University of Porto

External Host Supervisor

Dr. Paula Sampaio, Platform Head, Institute for Research and
Innovation in Health



To my family

Acknowledgements

For the last nine months there were few moments in which the work presented in this dissertation wasn't on my mind. Even in time spent under the sun or on nights out dancing, part of it still lingered in the background. This little endeavor has taken up a significant part of my life. There were feelings of success at every step, but also moments in which the task at hand, the objectives that I set out for myself, seemed too much to handle. However, throughout these nine months, throughout these six years, I was fortunate to have special people by my side that have never allowed me to stop. No matter how hard I try, no words will ever be able to capture the depth of my gratitude. Nevertheless, I will try.

First, I would like to thank my supervisors, Dr. Paulo Marques and Dr. Paula Sampaio. Thank you, Professor, for teaching me so much over these years. Your guidance has been instrumental in bringing this course to its conclusion. Thank you, Dr. Paula Sampaio, for this opportunity and for granting me every tool and all the freedom I needed to express myself in this project.

To Maria Azevedo. Thank you for every single time I interrupted you to discuss this project. You never wavered. Better yet, it was always with excitement that you responded to my turmoil. Your patience, guidance, and kindness were some of the most significant pieces of this puzzle. If I hadn't shared that desk with you, none of this would have been possible. For that I will always be grateful. Thank you.

To all my friends. To the ones that have been around since I remember existing. You have seen me grow. Have grown right beside me. You are my home and forever will be. To the ones that received me in this city with so much warmth. To the ones who wore a black cape with me. Thank you for all the moments we shared, all the coffee breaks, classes, and all the nights, studying and not. We have made it, together.

To Francisca, for being my safe space, my haven, my guardian angel. You have always supported me in all my ideas, ventures, and struggles. Your tenderness has always spoken louder than anything else. With you, I am my happiest.

Lastly, to my family. To my grandmother, who for all my life has received me in her bedroom with the same elation. I will always be waking you to give you your yogurt. To my brother. For everything you taught me, everything you showed me, and for instilling in me

the desire to learn. After you went on your journey and started your studies, seeing you from a distance has given me the greatest lesson on perseverance I could have asked for. We are now closer than ever. Please continue teaching me everything. To my parents, for always believing in me, pushing me to do better, and teaching me how to roam in this life. You are my two strongest pillars. Everything else was built because of you. All your sacrifices have made me reach this far. Thank you for granting me everything I could have asked for.

Resumo

Esta dissertação apresenta a conceção e o desenvolvimento do *VitaSlice*, um *software* desenvolvido para controlar um Microscópio de Fluorescência por Folha de Luz (*Light Sheet Fluorescence Microscope* - LSFM) construído de raiz, de forma personalizada. O software foi desenvolvido na plataforma científica *Advanced Light Microscopy* (ALM) do i3S - Instituto de Investigação e Inovação em Saúde, e está otimizado para a utilização num ambiente de centros de investigação com acesso a recursos partilhados. Especificamente, foi construído de forma a proporcionar a acessibilidade de um sistema personalizado a utilizadores sem conhecimentos óticos ou técnicos.

Embora os sistemas LSFM comerciais ofereçam um desempenho robusto e facilidade de utilização, o seu custo pode ultrapassar os seis dígitos, o que os coloca fora do alcance financeiro de muitos centros de investigação. Em contrapartida, o desenvolvimento de um sistema personalizado reduz significativamente o custo, através do uso de dispositivos de *hardware* individuais e *software* de código aberto. Desta forma, este tipo de microscópio é altamente adequado para instituições que pretendam fornecer acesso a técnicas de imagiologia avançadas dentro de restrições orçamentais.

O microscópio apresenta a arquitetura de um *Digital Scanned Laser Light Sheet Microscope* (DSLMS). A sua aquisição de imagem volumétrica é efetuada utilizando uma amostra movível e um feixe de laser que é varrido num eixo, de forma a simular uma folha de luz plana. O sistema ótico do microscópio é composto por quatro lasers distintos, iluminação unilateral, duas câmaras para deteção, rodas de filtro, plataformas de translação e rotação e espelhos de varrimento galvanométrico. Sincronização temporal é obtida através do uso de uma placa de controlo ScanLab RTC5 como mestre, que envia pulsos elétricos TTL para o sistema de varrimento, câmaras e lasers. O sistema ótico foi alinhado com especial atenção à colineariedade dos feixes e ao correto posicionamento do seu plano focal, garantindo uma iluminação adequada da amostra e uma aquisição de imagens de qualidade.

Para assegurar a coordenação do hardware entre os dispositivos, foi concebida e implementada uma caixa de comunicação personalizada. Foi construída para redistribuir os sinais elétricos da placa RTC5 para as entradas de modulação digital dos quatro lasers

e para as entradas de acionamento externo das câmaras. O circuito no seu interior foi construído utilizando um circuito integrado que corresponde a uma matriz de transístores *Darlington* para amplificação da corrente e, à saída, resistências de divisão de tensão para adaptação do sinal. A caixa dispõe de cinco terminais de saída BNC para acionamento dos lasers e câmaras. O seu sucesso foi validado através da medição direta do sinal das saídas utilizando um osciloscópio, bem como da ativação bem-sucedida da emissão dos lasers e do acionamento da exposição da câmara em resposta aos sinais emitidos.

Foi desenvolvido um software em *Python* para controlar o microscópio. Foi construído de raiz, comunica com cada dispositivo do microscópio através de interfaces de programação de aplicações de alto nível e inclui uma interface gráfica modular, baseada em *widgets*, construída com a biblioteca *PySide6*. A incorporação do *napari* permite a visualização de imagem em tempo real, e a utilização do formato OME-Zarr para os ficheiros de imagens assegura o armazenamento em conformidade com as normas modernas de bioimagem, incorporando simultaneamente metadados.

O software permite a aquisição de imagens em tempo real, volumétrica, multicanal e *time lapse*. O seu sucesso técnico foi validado através de uma série de aquisições de amostras biológicas relevantes, tais como células primárias de micróglia e oligodendrócitos encapsuladas em hidrogéis e larvas de peixe-zebra. O sistema demonstrou uma rápida obtenção de imagens, com aproximadamente 470 imagens por minuto em aquisições volumétricas, e uma elevada estabilidade durante longos períodos de aquisição. A sua facilidade de utilização foi também aprovada por comentários dos utilizadores da ALM.

O *VitaSlice*, ao permitir o controlo de um sistema de microscopia complexo através de uma abordagem intuitiva, mostra o potencial que as ferramentas personalizadas modernas, construídas com engenharia precisa, podem oferecer para garantir o acesso a técnicas de microscopia avançadas. Desta forma, o trabalho apresentado nesta dissertação contribui para o avanço da instrumentação científica acessível.

Palavras-chave: Microscopia *Light Sheet*, Aquisição de Imagem, Integração de *Hardware*, Desenvolvimento de *Software*.

Abstract

This dissertation presents the design and development of VitaSlice, a software developed to control a custom-built Light Sheet Fluorescence Microscope (LSFM). The software was developed at the Advanced Light Microscopy (ALM) scientific platform of i3S – Institute for Research and Innovation in Health - and is optimized for use in a shared facility environment. Specifically, it is tailored to provide accessibility of a custom-built system to users without optical or technical expertise.

While commercial LSFM systems offer robust performance and ease of use, their cost can exceed six figures, often putting them beyond the financial reach of some research institutions. In contrast, the development of a custom system significantly reduces the cost by employing individual hardware devices and open-source software. For this reason, this type of microscope is highly suitable for institutions that intend to provide access to advanced imaging techniques within budget constraints.

The microscope is based on the Digital Scanned Laser Light Sheet Microscope (DSLM) architecture. Its volumetric imaging is done using a movable sample and a scanned laser beam to simulate a planar light sheet. The microscope's optical system is composed of four distinct lasers, one-sided illumination, dual-camera detection, filter wheels, translation and rotation stages, and galvanometric scanning mirrors. Time synchronicity is achieved using a ScanLab RTC5 control board as a master, which sends TTL signals to the scanning system, cameras, and lasers. The optical system was aligned with careful attention to beam collinearity and proper focal plane positioning of the beams, ensuring appropriate sample illumination and image acquisition, enhancing the resulting quality.

To ensure hardware coordination between the devices, a custom-made communication box was designed and implemented. It was built to handle the electrical redistribution of the signals from the RTC5 board to all four lasers' digital modulation inputs and the cameras' external trigger inputs. The internal circuit was built using a Darlington transistor array integrated circuit for current amplification and voltage-dividing resistors for signal adaptation. The box provides five BNC output terminals for lasers and camera triggering. Its success was validated through direct signal measurement using an oscilloscope, as well as the successful activation of laser emission and camera exposure triggering as a

response to the emitted signals.

A Python-based software was developed to control the microscope. It was built from scratch, communicating with each microscope device through high-level Application Programming Interfaces (APIs), and employing a modular, widget-based graphical user interface built with the PySide6 library. The embedding of napari enables real-time image display, and the use of the OME-Zarr format for the image files ensures storage in compliance with modern bioimaging standards, while also employing built-in metadata.

The software supports real-time imaging, multi-channel volumetric imaging, and time-lapse acquisition. Its technical success was validated through a series of imaging experiments, including the acquisition of relevant biological samples, such as microglia and oligodendrocyte primary cells encapsulated in hydrogels, and zebrafish larvae. The system demonstrated high throughput of image data, with approximately 470 frames per minute in volumetric acquisitions, and high stability over long acquisition periods. Its usability was also approved by the facility users' feedback.

VitaSlice, by allowing control of a complex microscopy system through an intuitive approach, showcases the potential of modern, custom-built tools and precise engineering in granting access to advanced microscopy techniques. In this way, the work presented in this dissertation makes a positive contribution to the field of accessible scientific instrumentation.

Keywords: Light Sheet Microscopy, Image Acquisition, Hardware Integration, Software Development.

Table of Contents

Acknowledgements	ii
Resumo	iv
Abstract	vi
Table of Contents	viii
List of Tables	xi
List of Figures	xii
List of Abbreviations	xiii
Preface	1
1. Background & State of the Art	3
1.1. Fluorescence in Microscopy	3
1.2. Light Sheet Fluorescence Microscopy	6
1.2.1. Sheet of Light	7
1.2.1.1. Optical Properties	7
1.2.1.2. Light Sheet Shaping	8
1.2.2. Common Hardware in LSFM	9
1.3. State of the Art Systems	10
1.3.1. Commercial vs. Custom and Open-Source Systems	10
2. ALM Microscope Design	15
2.1. Microscope Devices	15
2.1.1. Laser Box	15
2.1.2. Scanning Mirrors	17
2.1.3. Linear and Rotation Stages	17
2.1.4. Filter wheels	18
2.1.5. Cameras	19
2.2. Full System Architecture	20
2.2.1. Reflection Optics	21
2.2.2. Immersion Chamber as a Positioning Reference	22
2.2.3. Illumination Path	23
2.2.4. Image Acquisition	25
2.3. Control & Coordination	27
2.3.1. Master Control via RTC5 and Synchronization Strategy	27
2.3.2. Hardware Development for Signal Distribution	28

3. Software Development.....	31
3.1. Design Requirements and Tools.....	31
3.1.1. Python as the Programming Language.....	32
3.1.2. Integration of napari.....	32
3.1.3. OME-Zarr for Image Storage.....	33
3.2. Software Architecture	33
3.3. Implementation	35
3.3.1. User Interaction.....	35
3.3.2. Individual Device Control Widgets.....	36
3.3.2.1. Filter Wheels Widget	36
3.3.2.2. Lasers Widget	37
3.3.2.3. Stages Widget.....	39
3.3.2.4. Scanner Widget.....	41
3.3.2.5. Cameras Widget.....	42
3.3.2.6. Integration of napari and Image Handling.....	44
3.3.3. Advanced Control: Automated Acquisition and File Management	45
3.3.3.1. File Manager Widget	45
3.3.3.2. Y-Stack Acquisition Widget	46
3.3.3.3. Acquisition Progress Window.....	49
3.3.3.4. Automated Acquisition Algorithms	50
3.3.4. File Saving and Metadata Recording	53
3.3.4.1. Zarr Array allocation and Frame writing.....	53
3.3.4.2. Metadata Integration	54
3.3.4.3. Settings File.....	54
4. Results - System Capabilities	56
4.1. Sample Preparation	56
4.1.1. Hydrogel Preparation	56
4.1.2. Zebrafish Larvae Preparation	57
4.2. Image Acquisition Results	57
4.2.1. Hydrogel Imaging	57
4.2.2. Zebrafish Larvae Imaging	58
4.2.3. Live Zebrafish Time-lapse.....	60
5. Conclusions & Future Work	62
5.1. Discussion & General Conclusions	62
5.2. Future Work	64
A. Filters available on the microscope.....	67

B. Supplementary Multimedia.....	68
B.1. ALM light sheet microscope system photographs.....	68
B.2. ALM light sheet microscope device photographs.....	69
B.3. ALM light sheet microscope subsystem photographs.....	70
C. External Resources	71
C.1. VitaSlice github repository	71
C.2. Recording of imaging experiment.....	71
D. Acquisition settings file example	72
References.....	73

List of Tables

1.1. Comparison of different commercial LSFM systems	12
2.1. Beam characteristics of the OBIS lasers	16
2.2. Performance parameters of the SCANcube 7 galvanometric scanner	17
2.3. Performance parameters of the PI linear and rotation stages	18
2.4. Specifications of the Hamamatsu ORCA-Fusion C13440-20CU Camera	19
3.1. Comparison between the available acquisition algorithms.....	52
A.1.Filter wheel 1 configuration	67
A.2.Filter wheel 2 configuration	67

List of Figures

1.1. Jablonski diagram and spectra of the fluorescence phenomenon	3
1.2. Direct and indirect immunofluorescence techniques	4
1.3. Illustration of the Gaussian beam	7
1.4. Illustration of the SPIM and DSLM architectures	9
1.5. Images of commercial LSFM systems	11
1.6. Open-Source and custom LSFM systems with examples of acquired images	13
2.1. Microscope's Coherent Laser Devices	16
2.2. Microscope's SCANcube 7 scanner and RTC5 control board.....	17
2.3. Microscope's PI Linear and Rotation Stages.....	18
2.4. Microscope's Zaber FWR-E Filter Wheel.....	19
2.5. Microscope's Hamamatsu ORCA-Fusion C13440-20CU Camera	19
2.6. Illustration of the light sheet microscope.....	21
2.7. Image of the stage system and schematic of the reference axes	23
2.8. Illustration of the illumination system's working principle	23
2.9. Illustration of the optical cage system	24
2.10. Illustration of the scanner holder	25
2.11. Illustration of the image formation in the image acquisition system	26
2.12. Schematic illustration of the developed circuit.....	28
2.13. Images of the communications box.....	29
3.1. Schematic illustration of the software's architecture.....	34
3.2. Display of the software main window	36
3.3. Display of the Filter Wheels Widget	37
3.4. Display of the Lasers Widget	38
3.5. Display of the Stages Widget.....	39
3.6. Display of the Scanner Widget.....	41
3.7. Display of the Camera Widgets.....	42
3.8. Display of the napari viewer	44
3.9. Display of the File Manager Widget.....	45
3.10. Display of the Y-Stack Acquisition Widget and Acquisition Progress Window.....	47
4.1. Maximum intensity projection images of the microglia and oligodendrocyte hydrogel sample	58
4.2. Maximum intensity projection images of zebrafish larvae	59
4.3. Single slice images of zebrafish larvae.....	59
4.4. Time-lapse acquisition images of a zebrafish larva	61
B.1. Top view of the ALM light sheet microscope.....	68
B.2. Angled view of the ALM light sheet microscope	68
B.3. Images of the ALM light sheet microscope devices	69
B.4. Images of the ALM light sheet microscope's optical system	70

List of Abbreviations

ALM	Advanced Light Microscopy
LSFM	Light Sheet Fluorescence Microscopy / Microscope
GUI	Graphical User Interface
SPIM	Selective Plane Illumination Microscopy
sCMOS	scientific Complementary Metal–Oxide–Semiconductor
FOV	Field of View
SNR	Signal-to-Noise Ratio
NA	Numerical Aperture
DSLM	Digital Scanned Laser Light Sheet Microscopy / Microscope
CCD	Charge-Coupled Device
EMCCD	Electron Multiplying Charge-Coupled Device
WD	Working Distance
CW	Continuous Wave
RMS	Root-Mean-Square
PI	Physik Instrumente
IC	Integrated Circuit
API	Application Programming Interface
DLL	Dynamic Link Library
ROI	Region-of-interest
FEP	Fluorinated Ethylene Propylene
OME	Open Microscopy Environment
AI	Artificial Intelligence

Preface

Dissertation overview

The developed work took place at i3S – Institute for Research and Innovation in Health, more specifically, at the Advanced Light Microscopy (ALM) scientific platform [1]. The platform provides advanced imaging services to researchers, helping them obtain high-quality images of biological samples and assisting with subsequent image processing and analysis. The platform addresses a wide range of experimental needs by offering technical expertise and access to state-of-the-art optical microscopy equipment.

This dissertation is divided into five chapters. Chapter 1 provides background on fluorescence microscopy and reviews the current state of the art in both commercial and custom Light Sheet Fluorescence Microscopy (LSFM) systems. Chapter 2 describes the architecture and hardware components of the custom LSFM system developed at ALM, detailing the optical layout and synchronization strategies. Chapter 3 focuses on the development of the control software, covering both software architecture and practical implementation. Chapter 4 presents biological imaging experiments as results, validating the technical success and performance of the developed system. Finally, Chapter 5 offers a discussion of the obtained results, general conclusions of the developed system, and potential future improvements.

Objectives

The primary objective of this dissertation is the development of software designed to control a custom-built light sheet fluorescence microscope. It is important to note that the system is intended for operation within ALM - a shared scientific platform. Its main users are researchers with backgrounds in the life sciences and may not possess advanced knowledge of the optics and digital hardware control that constitute such a system.

To address this, the software should provide a Graphical User Interface (GUI) that offers a user-friendly experience, abstracting from the complexity of the microscope's architecture, much like the commercial systems available in the platform.

The software should then bridge the gap between complex instrumentation and accessible experimental workflows, enabling users to operate the microscope efficiently without relying on expert technical assistance.

1 Background & State of the Art

1.1 Fluorescence in Microscopy

Biological imaging greatly benefits from fluorescence. This is because fluorescence-based experiments offer high-labeling specificity, excellent sensitivity, quantitative analysis, and high resolution. They can also be performed on live or fixed samples.

To achieve this, fluorescent dyes or proteins are commonly used. These are molecules capable of absorbing light and subsequently emitting light at a longer wavelength. They act as tags, allowing the identification and characterization of cells, subcellular structures, and molecules, tracking gene expression, and visualizing specific proteins.

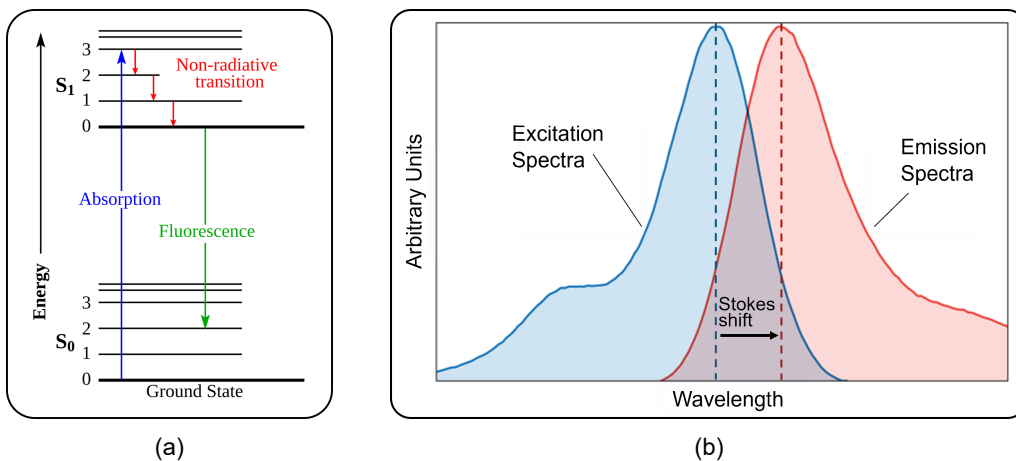


FIGURE 1.1: (a) Jablonski diagram that showcases the absorption and emission of photons by electrons in matter, from Ref. [2]. (b) Graphical representation of the Stokes shift seen in the excitation and emission spectra, adapted from Ref. [3].

The phenomenon of fluorescence was first described by Sir George Stokes in 1852 [4]. It is a subtype of photoluminescence, which occurs naturally in certain materials and organisms. Fundamentally, when a photon of light is absorbed by a molecule's electron, it is elevated to a higher energy level. Since the excited state is generally unstable, the electron rapidly returns to the lower energy state, typically within 1-10 nanoseconds.

As illustrated in Figure 1.1a, upon the initial photon absorption, the electron might lose energy through vibrational relaxation, which leads to the emission of a photon with less energy and a longer wavelength. The difference between the maximum excitation and

emission is known as the Stokes shift [5]. A graphical representation of the difference between both spectra is seen in Figure 1.1b. Moreover, the redshift is consistent with the Planck relation in equation 1.1, which describes the inverse proportionality between the energy of the photon and its wavelength [6].

$$E = \frac{hc}{\lambda} \quad (1.1)$$

It was in the 1960s that the green fluorescent protein (GFP) was discovered in *Aequorea victoria* jellyfish [7]. Since then, multiple fluorescent proteins and fluorophores have been developed, now allowing the coverage of almost the entire visible light spectrum. Some are small-molecule dyes, such as those from the Alexa Fluor or Cyanine families [8], which offer high quantum yields and narrow spectral bands. These characteristics make them well suited for staining in fixed samples. Their chemical structure also facilitates straightforward conjugation to antibodies, making them compatible with immunofluorescence protocols [9].

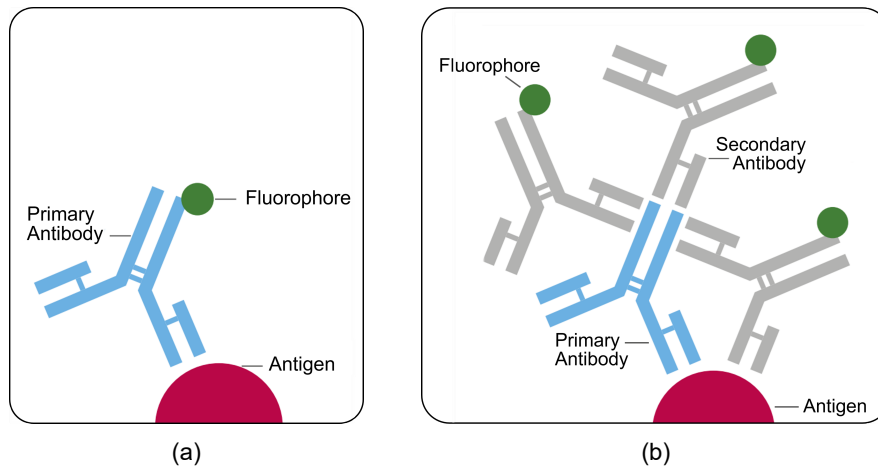


FIGURE 1.2: Illustration of immunofluorescence techniques. (a) Direct method, where the fluorophore is attached to the primary antibody targeting the antigen. (b) Indirect method, where multiple secondary antibodies, conjugated to the fluorophore, are bound to a primary antibody already attached to the target. Adapted from Ref. [10].

Another class of fluorophores includes the genetically encoded fluorescent proteins. These are introduced into the organism's genome via recombinant DNA techniques. Such proteins include GFP (green fluorescent protein) and its derivatives, such as mCherry or YFP (yellow fluorescent protein), and they enable the endogenous labeling of specific proteins [11]. This facilitates live-cell imaging of dynamic intracellular processes without the need for invasive staining protocols.

Equally important are the immunofluorescence protocols, which were developed for the precise localization of intracellular components labeled with specific fluorophores, while minimizing damage to biological samples. They can be implemented through direct labeling, where the fluorophore is attached to the primary antibody targeting the antigen, or through indirect labeling, where often multiple secondary antibodies, conjugated to the fluorophore, are bound to the primary antibody already attached to the target, as seen in Figure 1.2. While the direct method is simpler and more affordable, the indirect method usually yields a stronger signal due to the amplification of fluorophores bound to a single primary antibody [12].

In parallel with advances in fluorescence tools, the depth of biological research has benefited from improvements in the optical instrumentation used for imaging.

The widefield fluorescent microscope can be considered the fundamental imaging technique used in the field of biological sciences. Typically, it includes a broadband light source for excitation (such as a mercury lamp or light-emitting diodes), an excitation filter that is used to narrow the light source's spectrum, a dichroic mirror that reflects it, and an objective lens to focus the light onto the sample. The red-shifted fluorescent signal is collected by the same objective and is now transmitted through the dichroic mirror. An emission filter further selects the fluorescent light before it reaches the detector, typically a camera [13].

This configuration enables a swift workflow for its user, fast imaging across a wide field of view, and is well suited for observing thin samples. However, its limitation is the lack of optical sectioning, as the entire volume is illuminated simultaneously, including out-of-focus planes, which reduces image contrast and resolution, especially in thicker samples [14].

Laser scanning confocal fluorescence microscopy addresses the primary limitation of the widefield microscope, delivering sharp images [15]. In this system, excitation is performed using a laser source that emits highly monochromatic and collimated light. The beam is steered through a pair of galvanometric scanning mirrors and focused by the objective onto a single diffraction-limited point in the sample. The emitted fluorescent signal takes the opposite path and is directed through a pinhole aperture located at a plane optically conjugate to the objective's focal plane. The pinhole is the structure responsible for further enhancing optical sectioning by spatially filtering the signal, allowing mainly in-focus light

to reach the detector. This configuration, however, has its own drawbacks. Since the image is acquired point by point, the acquisition process is relatively slow. Additionally, the high intensity of the laser light that passes through the entire volume of the sample can lead to photobleaching and phototoxicity, especially when working with live samples.

To overcome some of the limitations of conventional laser scanning confocal systems, such as slow acquisition speed or photodamage, alternative techniques with optical sectioning properties have been developed. These include multi-photon microscopy [16], spinning-disk confocal microscopy [15], and finally, light sheet fluorescence microscopy.

1.2 Light Sheet Fluorescence Microscopy

Light Sheet Fluorescence Microscopy (LSFM) is a general name for a constantly growing family of planar illumination techniques. Also known as Selective Plane Illumination Microscopy (SPIM), it has its conceptual roots in the ultramicroscope from the early 20th century, which used planar side illumination for particle visualization [17]. The next step came in 1993 with Voie et al.'s orthogonal plane fluorescence optical sectioning. It demonstrated optical sectioning in bulk specimens such as the guinea pig cochlea [17]. The landmark 2004 SPIM design by Huisken et al. introduced a dual-objective configuration – a dedicated illumination objective to project a light sheet into the sample and an orthogonally placed detection objective to capture fluorescence. That arrangement enabled real-time subcellular imaging in live embryos.

Fundamentally, LSFM techniques are made possible by decoupling the illumination and detection optical paths, thereby optimizing the photon-collection efficiency of the instrument [18]. Illumination is predominantly done using laser beams, and a light sheet is formed by shaping them into a hyperbolic "sheet" or approximated by scanning a focused, low numerical aperture (NA) in the lateral directions, analogous to laser scanning microscopes. Detection is performed along a different axis, often orthogonally, resulting in high-contrast images with minimal out-of-focus blur.

In contrast with epifluorescent techniques, where excitation and detection are done using the same objective and light path, and where the total volume of the sample is illuminated, light sheet illumination doesn't unnecessarily excite significant out-of-focus fluorescence planes that would contribute to out-of-focus light, degrading the quality of the in-focus

signal [18]. With LSM, lower illumination intensities can be used in conjunction with planar illumination to enhance the signal-to-noise ratio (SNR) with minimal exposure.

Additionally, axial confinement of illumination results in reduced photobleaching and phototoxicity, which allows long-term imaging over days or even weeks [18]. Nonetheless, LSM can be challenging to implement, requiring complex instrumentation such as multiple objectives, non-standard sample preparation protocols, and intricate alignment procedures. Since there is a rapid flow of images, a planned workflow is necessary to manage the massive amounts of data that are produced.

1.2.1 Sheet of Light

1.2.1.1 Optical Properties

As mentioned earlier, there are several methods to form a light sheet. In the SPIM technique, a static planar light sheet is used. In contrast, different techniques employing a scanned beam to approximate a light sheet over time can be employed.

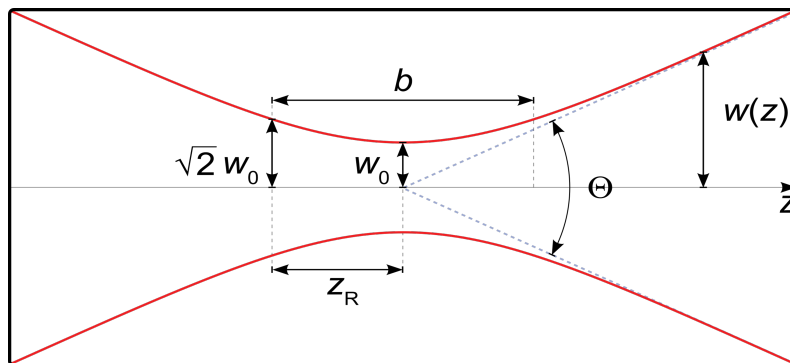


FIGURE 1.3: Illustration of the Gaussian beam, displaying the relevant w_0 , $\sqrt{2}w_0$, and b parameters. Adapted from Ref. [19].

First, it is important to establish that it's theoretically impossible to create a perfectly planar light sheet [17]. It can only be approximated, and only within a limited range. Axial resolution is limited by the thickness of the light sheet and the NA of the detection objective. If beam propagation happens in the Z direction, both static and scanned light sheets have a hyperbolic profile in the XY -plane, with the beam waist w_0 specifying the thickness of the most tightly focused point of the beam. It depends on the focal length of the illumination optics, the wavelength of light, and the diaphragm of the illumination optics. Equation 1.2 displays these relations.

$$w_0 = \frac{1.4 \cdot f \cdot \lambda}{2 D} \quad (1.2)$$

Of particular concern is the confocal parameter b of the light sheet, which defines the distance in which the sheet approximately provides near-homogenous planar illumination. It is defined by the Rayleigh range Z_R , which is the distance from the center of focus to the point where the beam waist has increased by a factor of $\sqrt{2}$. The confocal parameter and beam waist relationship is given by the equation 1.3.

$$b = 2 \cdot Z_R = \frac{2\pi w_0^2}{\lambda} \quad (1.3)$$

Thus, increasing the confocal parameter by creating a larger light sheet directly results in a thicker beam waist, limiting axial resolution. An important caveat is that these equations refer to the propagation of light in air, but in a medium with refractive index n , the confocal parameter is given by equation 1.4.

$$b_n = \frac{2\pi w_0^2}{\lambda} \cdot n \quad (1.4)$$

Consequently, it is possible to extend the light sheet and improve axial resolution by propagating the beam in higher refractive index materials, such as water ($n = 1.33$).

1.2.1.2 Light Sheet Shaping

Some LSFM techniques employ a planar light sheet for illumination. They are achieved by using a single cylindrical lens that modulates the incident beam onto a light sheet.

In the case of a scanned beam, to obtain volumetric data in the form of Z -stacks, usually the sample is scanned axially. More recently, in 2008, the Digital Scanned Laser Light Sheet Microscope (DSLM) was developed by Philipp Keller [17]. It utilizes a standard Gaussian laser beam rather than a light sheet and scans the beam laterally to simulate a planar light sheet. Then, using an f -theta lens, the angular movement of the beam is converted into an axial translation. The beam is thus scanned through the sample while the camera is exposing for an image to be acquired. Z -stacks can be obtained by moving the sample through the focal plane.

1.2.2 Common Hardware in LSFM

In practice, fully assembling an LSFM is a complex task, largely due to its differences in optical system architecture from traditional microscope designs. Nonetheless, to implement an LSFM, regardless of the architecture, there is always a set of necessary hardware.

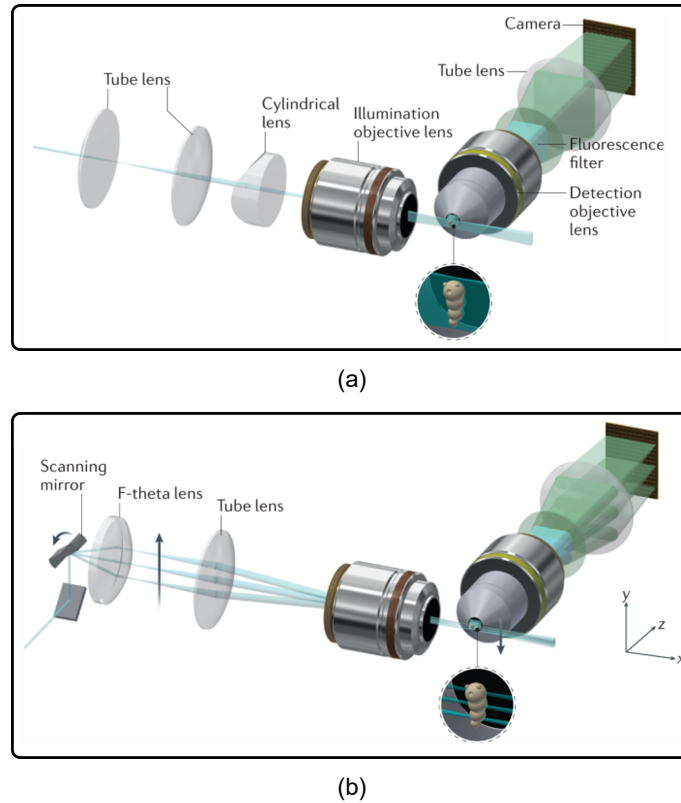


FIGURE 1.4: Illustrations of the different architectures in image acquisition. (a) SPIM architecture, employing a cylindrical lens to form the light sheet. (b) DSLM architecture, which employs a set of galvanometric mirrors to scan the sample for illumination. Both adapted from Ref. [18].

In a broader perspective, the camera is perhaps the most vital component of any LSFM system. The use of charge-coupled devices (CCD) and electron multiplying CCDs (EMCCD) is the industry standard for most imaging systems requiring molecule-level sensitivity. The most common practice in LSFMs, however, is the use of scientific complementary metal-oxide-semiconductor (sCMOS) devices. Their advantages include moderate cost, larger sensor sizes compared to CCDs and EMCCDs, and high imaging speeds as fast as 100 Hz. A larger sensor can make the difference between imaging the whole sample or not, which would then require more complicated workflows such as multiple acquisitions and the use of image-stitching algorithms. Furthermore, rapid image acquisition is crucial for LSFM to realize its full potential, especially in live imaging, where events occur in three dimensions at a very high rate [17]. An increasingly common approach in DSLMs

includes the rolling shutter ability of an sCMOS camera, synchronized with the scanning of the beam through the field of view (FOV) to act as a confocal slit aperture, filtering out-of-focus light and enhancing axial resolution, matching confocal system's performance.

Another essential component is the objective lens, for which several factors must be considered in its selection. The sample volume intended for the system to image is perhaps the most crucial, since it determines the optimal FOV, working distance (WD), and depth of field. Usually, detection objectives for LSFM are low to mid NA, ranging from 0.2 to 1.1. They can be dry or water immersion objectives and have a longer WD. If used as an illumination objective, it should have 2-10 $\times$ magnification and an NA of 0.1 to 0.3 [17].

The light source for LSFM is almost always a laser. In planar light sheets, only about 5% of the laser intensity is conserved due to beam-shaping optics, while in the scanning counterpart, about 95% of the original intensity reaches the sample.

As was already mentioned, the amount of generated data in an experiment using this technique can be massive, often many terabytes. For this reason, a data management pipeline that directly connects the LSFM computer to the analysis computer is crucial for handling these large quantities.

1.3 State of the Art Systems

Over the past years, the continuous growth of LSFM has led to a diverse range of system architectures, spanning from advanced commercial equipment to open-source and custom-made instruments. The various systems cater to the different needs of research institutions, whether that means accommodating unusual sample sizes, imaging modalities, or environmental conditions, striking different balances between accessibility, imaging quality, and experimental flexibility.

1.3.1 Commercial vs. Custom and Open-Source Systems

Commercial LSFM systems result from sustained advancements in optical design and engineering, providing standardized solutions that prioritize usability and integration over customizability.

Among the top platforms, the Zeiss Lightsheet 7 is an example of a stable and fully integrated system designed for long-term imaging of both live and cleared samples [22]. It

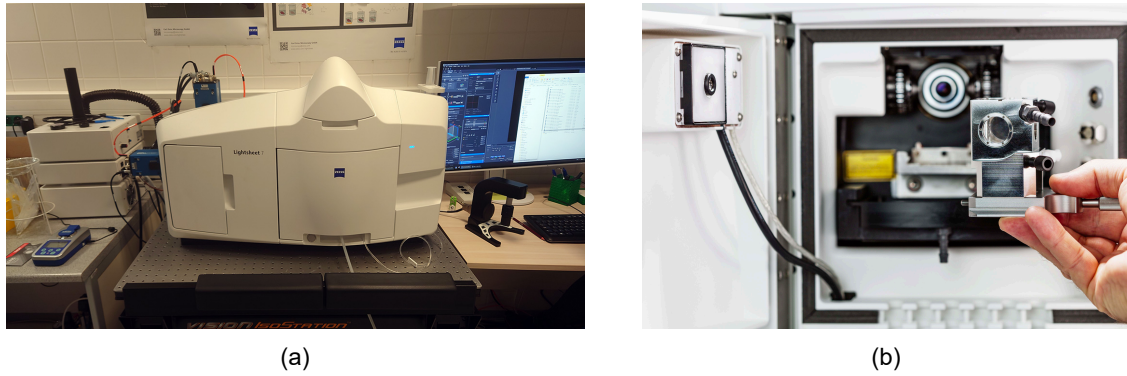


FIGURE 1.5: (a) Zeiss Lightsheet 7 at the Central European Institute of Technology, from Ref. [20]. (b) Interior of the Zeiss Lightsheet 7 with the chamber for objective and sample positioning, objectives, and light transmission camera used to easily find the sample, from Ref. [21].

features dual-sided illumination to reduce stripe artifacts and multi-view acquisition, providing isotropic resolution and efficient penetration in thick specimens. It accommodates large-volume samples through interchangeable objectives and refractive index-matched sample chambers. Additionally, its temperature, CO₂, and humidity are regulated to keep biological samples healthy and allow continuous long-term acquisitions with reproducible results.

The Zeiss Lattice Lightsheet 7 is another system that extends these capabilities by illuminating the sample with a structured lattice light sheet [23]. The lattice results from a Bessel beam modified using a spatial light modulator, changing its waveform and introducing a structured pattern. Axial resolution is greatly increased by this illumination, since it produces ultra-thin planes that significantly reduce the out-of-focus excitation. For wavefront correction, the system incorporates adaptive optics, which enhances image quality.

These systems typically provide proprietary software and optimized workflows, greatly increasing user accessibility. However, flexibility is often limited for hardware or software-level modifications, which could limit the execution of unconventional experimental setups.

Table 1.1 provides a comprehensive overview of the capabilities of various commercial LSFM systems. "Multi-Channel" refers to its ability to excite different fluorophores in the sample with distinct light sources. "Workflow Automation" refers to its ability to do sample manipulations, such as positioning, focusing, and filter and objective changes without manual intervention. "Photo-manipulation" refers to techniques that use focused light to locally alter the state of the sample.

TABLE 1.1: Capabilities of various commercial LSFM systems [22–26].

- Refers to an available feature.
- Refers to an unavailable feature.

	Live-Cell	Multi-channel	Fast (> 100 FPS)	High-Res. (< 200 nm)	Workflow Automation	Env. Ctrl.	Inverted Config.	Clearing Compat.	Adaptive Optics	Photo-Manipulation
Leica STELLARIS DLS	●	●	●	●	●	●	●	●	●	●
Zeiss Lightsheet 7	●	●	●	●	●	●	●	●	●	●
Zeiss Lattice Lightsheet 7	●	●	●	●	●	●	●	●	●	●
Bruker MuVi SPIM	●	●	●	●	●	●	●	●	●	●
Leica Viventis LS2	●	●	●	●	●	●	●	●	●	●

There are several commercially available configurations and architectures. Each architecture is specifically designed to enable the imaging of different types of samples, reflecting the diversity in LSFM systems. Examples of these are the Leica STELLARIS DLS, the Leica Viventis, and the Bruker MuVi [24–26].

Unlike commercial equipment, open-source and custom LSFM systems are typically designed and built by research groups or collaborative academic communities, offering improved flexibility and greater cost-efficiency.

Developed by a community-driven initiative at the Max Planck Institute of Molecular Cell Biology and Genetics, OpenSPIM is one of the most popular setups [32], providing access to the designs for building a SPIM system. The project includes detailed documentation, a parts list, and open-source control software. Image acquisition is achieved by focusing a thin laser light sheet onto a sample that is hanging in a sample chamber. Two-dimensional images of the illuminated slice are captured with a camera positioned perpendicularly. Volumetric data is obtained by moving the sample across the light sheet, with the possibility of rotating the sample and acquiring images from different angles.

Another popular configuration is mesoSPIM, originally developed by Fabian F. Voigt at the University of Zurich [33, 34]. Its goal was to image large, cleared samples, such as mouse brains. For increased resolution, an axially swept light sheet is synchronized with the camera readout, preventing light from out-of-focus planes from being detected. Dual-sided illumination and multi-view acquisition are also supported. Although their costs remain considerable compared to basic setups, they are still significantly more affordable than those of commercial systems.

Developers of LSFM systems often rely on flexible, open-source ecosystems to manage

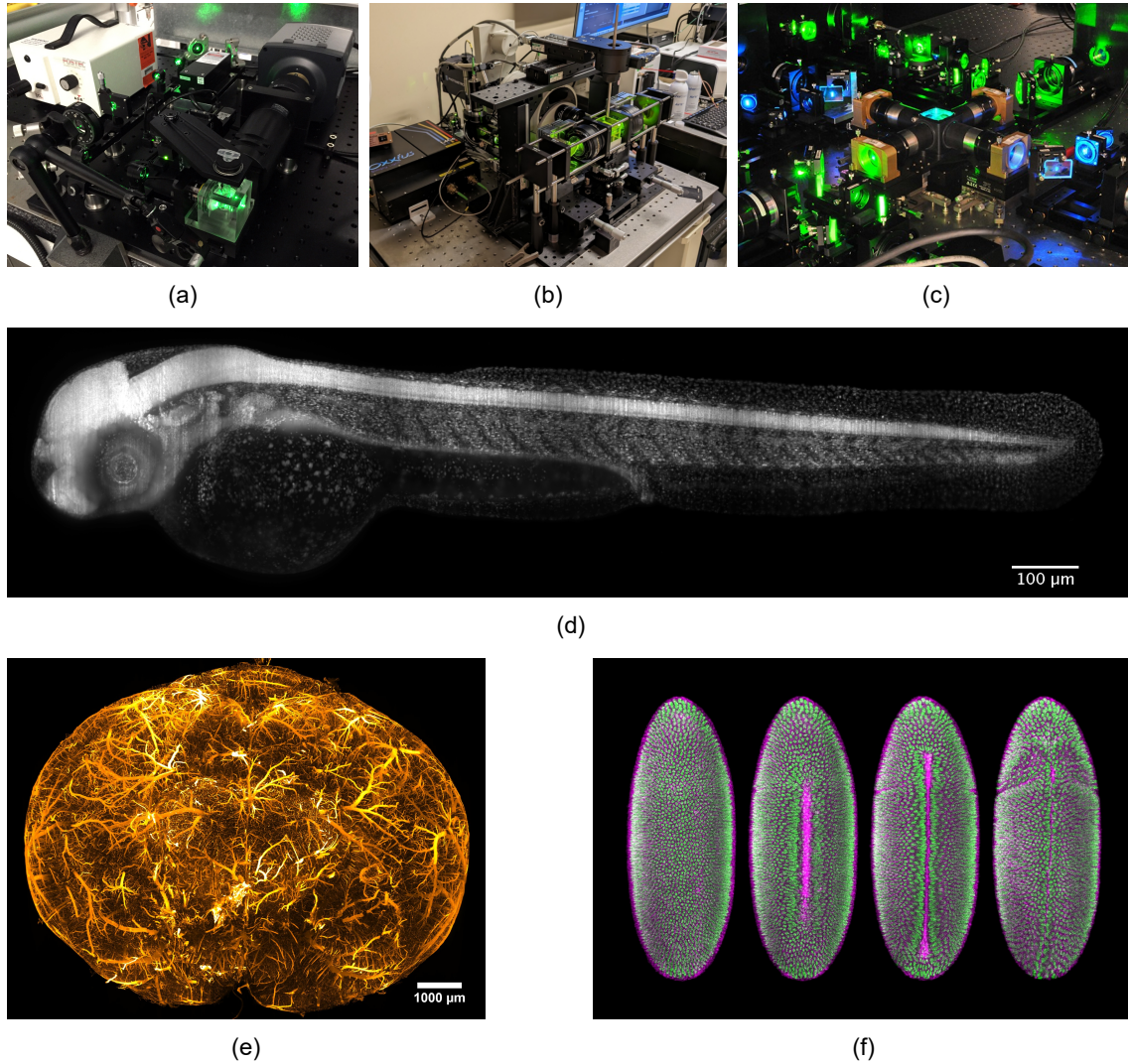


FIGURE 1.6: (a) OpenSPIM setup of the Florey Institute in Melbourne [27]. (b) mesoSPIM setup at the University of Pittsburgh [28]. (c) IsoView setup in the Janelia Research Campus [29]. (d) Zebrafish embryo captured using an OpenSPIM system [30]. (e) Mouse vasculature captured using a mesoSPIM system [31]. (f) Images of a *Drosophila* embryo development during 30 minutes [29]. While the image doesn't have a scale, *Drosophila* embryos are typically around 500 μm long.

available hardware. $\mu\text{Manager}$ is a robust open-source software platform for controlling microscope hardware and acquiring images [35]. It has support for a wide range of devices and provides a GUI for user interaction and scripting options, enabling automation for microscopy workflows. Its modular design allows researchers to add custom hardware components to their microscopes without requiring proprietary software. As a result, it became the central tool for most of the OpenSPIM setups [36].

A custom system that has expanded the scope of LSFM is IsoView [29], developed by

Keller and colleagues at the Howard Hughes Medical Institute's Janelia Research Campus. This microscope was designed to overcome the anisotropy of the traditional single-view light sheet setups. It features four orthogonally arranged light sheet arms, each with its own illumination and detection path. Serving as a valuable reference for custom system performance and innovation in LSFM, it is, however, not an open-source initiative.

Additionally, the developed software for LSFM systems should also be referenced, such as Cockpit [37]. It is a Python-based software project developed to provide intuitive, real-time control over complex custom microscopes. It features a GUI and supports the control of a wide range of hardware. In addition, it supports scripting for automated experiment workflows and is being increasingly adopted in research.

Open-source and custom LSFM initiatives address an important need in the research environment. Offering a sustainable alternative for laboratories that lack the funding for commercial solutions or the means to independently engineer proprietary systems, these systems demonstrate the increasing value of open-hardware initiatives in modern microscopy. By using community-based designs, modularity, and shared software development, custom LSFM systems provide robust alternatives to commercial ones.

2 ALM Microscope Design

The custom LSFM available at ALM is based on the DSLM architecture, featuring a single illumination path and two detection paths. In this chapter, a comprehensive overview of the microscope's constituent devices and their capabilities is provided. The chapter then presents the full system architecture and details the employment of specific communication and synchronization strategies between the devices during image acquisition.

Then, the full system architecture is presented, along with a detailed description of the interplay between devices during image acquisition, where different communication and synchronization strategies are employed.

2.1 Microscope Devices

All hardware devices are connected to a central control computer, which manages each device independently through various communication protocols and driver libraries. The control system enables programmatic manipulation of each device's parameters as well as their coordination.

2.1.1 Laser Box

The system incorporates four lasers enclosed in a Coherent OBIS LX/LS Laser Box [38–42], as displayed in Figure 2.1. The enclosure integrates the lasers into a single USB interface for computer control. The four lasers cover a wide excitation range that allows different fluorophores to be imaged. Their respective models are:

- OBIS LX 405 nm.
- OBIS LS 488 nm.
- OBIS LS 561 nm.
- OBIS LX 640 nm.

Each laser delivers up to 100 mW of output power and supports both continuous wave (CW) operation and external modulation, with analog (0-5 V) and digital (up to 150 MHz) connections [38].

TABLE 2.1: Beam characteristics of the OBIS lasers, from their data-sheet found in Ref. [43].

	λ (nm) $\pm$ tol.	Beam $\varnothing$ ($1/e^2$, mm)	Div. (mrad)	Noise RMS (%)	Stability (%) (8 h, $\pm 3^\circ\text{C}$)
OBIS LX 405 nm	405 ± 5	0.8 ± 0.1	< 1.0	≤ 0.05	< 2
OBIS LS 488 nm	488 ± 2	0.7 ± 0.05	< 1.2	≤ 0.25	< 2
OBIS LS 561 nm	561 ± 2	0.7 ± 0.05	< 1.2	≤ 0.25	< 2
OBIS LX 640 nm	640 ± 5	0.8 ± 0.1	< 1.3	≤ 0.05	< 2

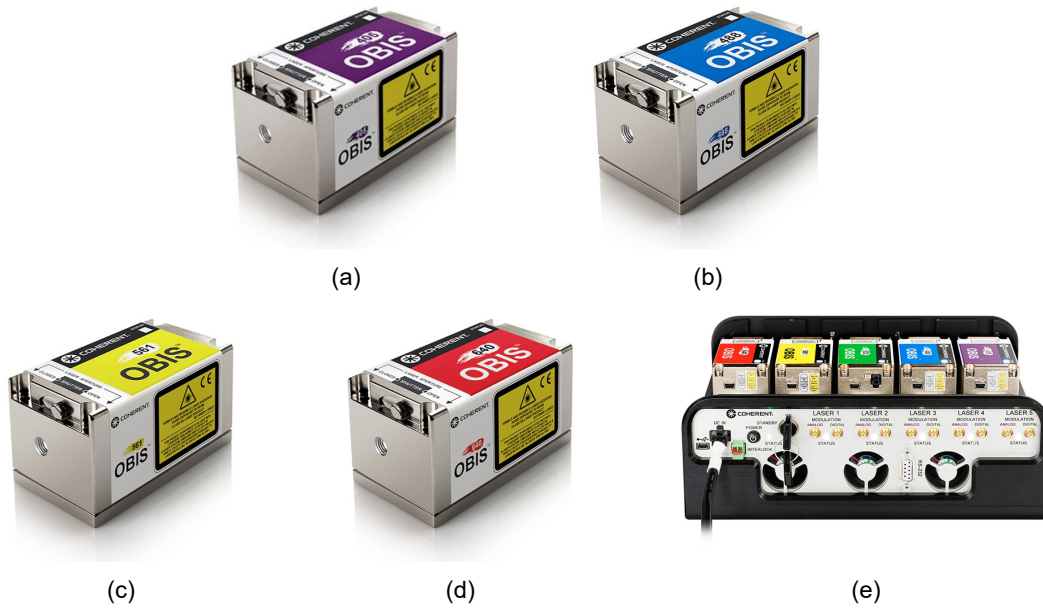


FIGURE 2.1: Laser devices in the light sheet microscope. (a) OBIS LX 405 nm, from Ref. [39]. (b) OBIS LS 488 nm, from Ref. [40]. (c) OBIS LS 561 nm, from Ref. [41]. (d) OBIS LX 640 nm, from Ref. [42]. (e) OBIS LX/LS Laser Box, from Ref. [38].

Table 2.1 summarizes the optical properties of the beams. Beam diameter values correspond to the $1/e^2$ beam width, defined as the diameter at which the intensity drops to 13.5% of its maximum. In addition, stability performance parameters are provided, including the root-mean-square (RMS) fluctuations of the power and long-term power variation over 8 hours, all of which are under 2%.

2.1.2 Scanning Mirrors

To digitally shape the illumination plane, a SCANcube 7 galvanometric scanner [44] is used to laterally scan the laser beam. The scanner outputs an angular beam deflection that is shaped by the optical system to approximate a planar light sheet.

Parameter	Value
Marking speed	2.5 m/s
Positioning speed	15.0 m/s
Repeatability (RMS)	$< 2 \mu\text{rad}$
Scan angle	$\pm 40^\circ$

TABLE 2.2: Performance parameters of the SCANcube 7 galvanometric scanner, from its datasheet found in Ref. [44].

The scanner is driven with a ScanLab RTC5 control board [45], which is directly connected to the control computer's motherboard via PCI Express. Its performance metrics are listed in Table 2.2, and both the scanner and the control board models are displayed in Figure 2.2.

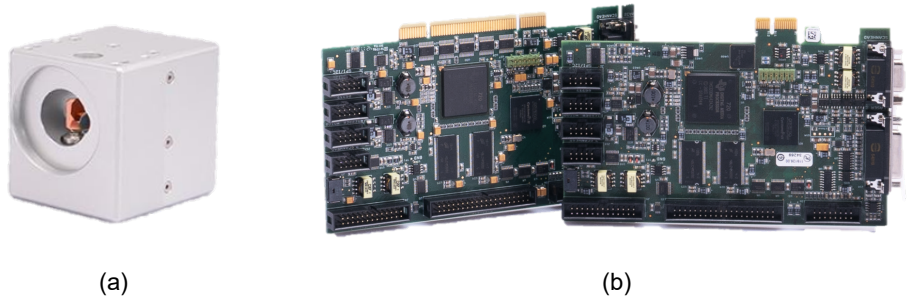


FIGURE 2.2: Galvanometric scanning system. (a) SCANcube 7, from Ref. [44]. (b) ScanLab RTC5 Control Board, from Ref. [46].

2.1.3 Linear and Rotation Stages

The system features a static illumination plane, with sample positioning being accomplished using a 3-axis linear translation stage system and a rotation stage. It comprises the following Physik Instrumente (PI) stages:

- Two PI M-110.1DG1 stages provide motion in the XY plane (5 mm $\times$ 5 mm travel range [47]).
- One PI M-112.1DG1 stage is used for the Z -axis movement, with a 25 mm range [47].
- A PI DT-34 rotation stage allows sample reorientation in yaw [48].

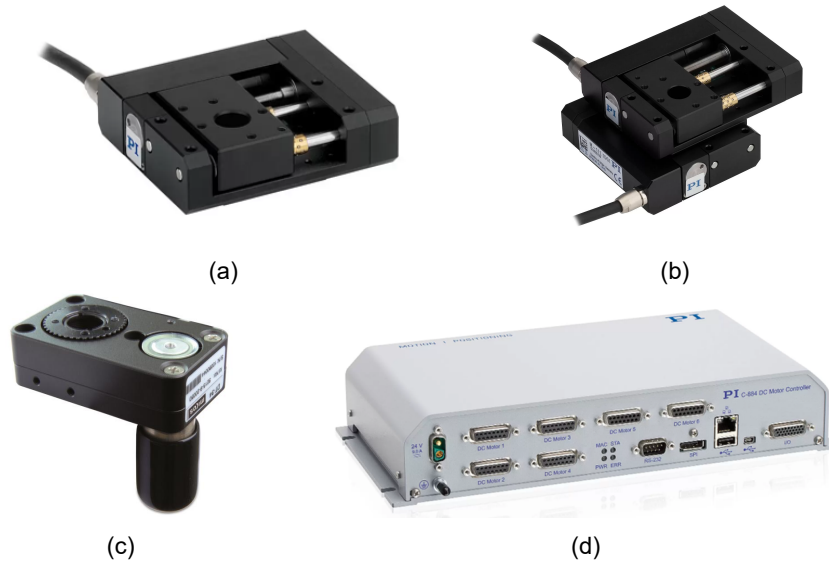


FIGURE 2.3: Linear and Rotation stages. (a) PI M-110.1DG1, from Ref. [49]. (b) PI M-112.1DG1 mounted on top of another linear stage, from Ref. [49]. (c) PI DT-34, from Ref. [48]. (d) PI C-884.4DC, from Ref. [50].

TABLE 2.3: Performance parameters of the PI M-110.1DG1, M-112.1DG1, and DT-34 positioning stages, from Ref. [47, 48].

	Range	Resolution	Unidirectional Repeatability	Max. Velocity
M-110	5 mm	0.0069 μm	0.25 μm	1 mm/s
M-112	25 mm	0.0069 μm	0.25 μm	1.5 mm/s
DT-34	360°	0.02°	0.04°	675°/s

All stages are controlled with a PI C-884 4DC motor controller, which connects to the control computer via USB [50]. Their performance metrics are shown in Table 2.3, along with the stages and motor controller in Figure 2.3.

2.1.4 Filter wheels

To filter the emission light, two Zaber FWR-E filter wheels [51] are used, one in each of the two detection paths. The device is illustrated in Figure 2.4. Each wheel accommodates ten 25 mm filters and enables high-speed switching (57 ms between adjacent filters), thereby minimizing delays during acquisition. Communication with the control computer is established via USB connection. The full list of available filters is provided in Appendix A, including their spectral characteristics.



FIGURE 2.4: Zaber FWR-E filter wheel, from Ref. [51].

2.1.5 Cameras

Two Hamamatsu ORCA-Fusion C13440-20CU sCMOS cameras [52] are used for fluorescence detection, shown in Figure 2.5. This model features a large sensor area, high quantum efficiency, and frame rates of 100 Hz, making it well-suited for LSFM applications, such as live-cell imaging and fast volumetric acquisition. Its specifications are summarized in Table 2.4.

Parameter	Specification
Chip size	13.312 mm $\times$ 13.312 mm
Format	2048 $\times$ 2048 (adjustable)
Pixel size	6.5 μ m $\times$ 6.5 μ m
Binning	1 $\times$ 1, 2 $\times$ 2, 4 $\times$ 4
Bit depth	16-bit, 12-bit, 8-bit

TABLE 2.4: Specifications of the camera, from Ref. [52].



FIGURE 2.5: Hamamatsu ORCA-Fusion C13440-20CU, from Ref. [53].

The camera supports both internal and external triggering, making it compatible with synchronized control. For exposure, two modes are available:

- Global shutter mode, in which the entire sensor is exposed simultaneously.
- Light sheet mode, in which a rolling shutter is used and synchronization with the beam scanning can be made, rejecting out-of-focus light and enhancing axial resolution.

Both cameras are connected to the control computer via a Camera Link interface, ensuring high-throughput data transmission.

The sensor features a $13.312 \text{ mm} \times 13.312 \text{ mm}$ sensor, which defines the physical area available for imaging and ultimately determines the FOV. Its format is 2048×2048 pixels, specifying the resolution as the number of discrete sensing elements in each dimension, and is adjustable to allow for cropped region-of-interest (ROI) readouts.

To improve SNR or reduce data volume, the camera supports multiple binning modes. Pixel binning is a capability of the camera to combine adjacent pixels by summing their values, trading off spatial detail for increased sensitivity.

Additionally, the camera can output image data in different bit depths. This parameter determines how many intensity levels can be assigned to each pixel. For example, a bit depth of 16 bits corresponds to 65536 possible intensity values per pixel (2^{16}). A higher bit depth increases the dynamic range, allowing finer differentiation between intensity values.

2.2 Full System Architecture

The microscope is composed of an architecture that can be conceptually divided into three distinct but interdependent systems: the reflection optics system, the illumination path system, and the image acquisition system. Each subsystem plays a critical role that, when integrated, enables precise light sheet imaging.

In this section, a description of the optical layout and mechanical configuration of each of these three subsystems is provided, detailing the beam path from the laser sources to the sample, as well as from the emitted fluorescence to the cameras. How the optical alignment considerations and the synchronization between devices ensure that illumination, scanning, and image acquisition occur in a coordinated manner is also explained.

A schematic representation of the complete system layout, including all major optical and mechanical components, is illustrated in Figure 2.6, and images of the full system can be seen in Appendix B.

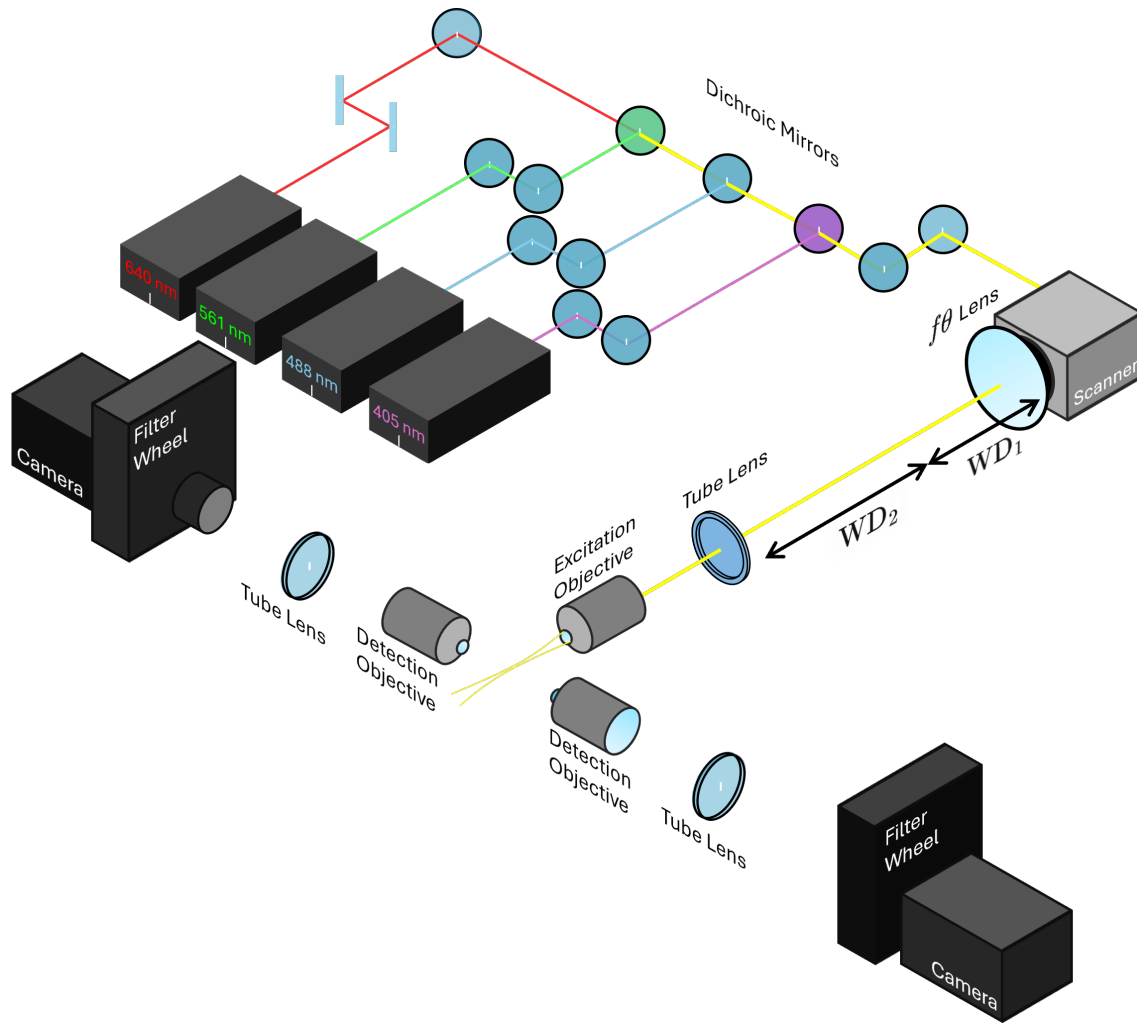


FIGURE 2.6: Illustration of the light sheet microscope, displaying the three main subsystems: the reflection optics system, the illumination path, and the image acquisition path. The diagram highlights the complete beam path from the laser sources to the theoretical sample position (not in display for clarity) and from the emitted fluorescence to the cameras.

2.2.1 Reflection Optics

The reflection optics system is responsible for aligning and steering the beams emitted by each of the four lasers so that they converge onto a common optical path. Its sole purpose is to ensure that all beams enter the scanning mirror's input aperture at the precise height of 7.4 cm above the optical table and travel parallel to the optical table surface.

To achieve this, each laser output is initially directed through a pair of broadband dielectric mirrors, which allow independent adjustment of beam height and angle. The first mirror steers the parallel output beam to decrease its height to 7.4 cm and redirects it to the second mirror, which corrects its parallel propagation.

Following the initial mirrors, the beams are sequentially combined using a series of long-pass dichroic mirrors selected according to the laser's emission wavelengths. The dichroics reflect each of their respective laser beams and transmit the other ones, merging each beam path into a single collinear path shared by all sources.

The quality of the optical alignment of this system is critical for image acquisition. Misalignment results in angular deviation or focal shift downstream, compromising the light sheet formation and illumination uniformity. For this reason, beam parallelism and correct entry height into the scanner's aperture were verified during the optical alignment procedure.

To facilitate the alignment procedure, each of the mirrors in the system was positioned to redirect the beam in 90° turns. Each laser was aligned individually, one at a time, starting with the one furthest from the scanner and progressing to the closest. To ensure the height of 7.4 cm, each beam was measured at multiple points along the optical path using a ruler, thus confirming its parallelism to the optical table.

The 90° reflections were optimized by taking advantage of the optical table's regular grid of screw holes as geometric reference points. For each reflection, the beam was steered in a direction directly above the optical table's screw holes. Two vertical laser safety guards were placed along the expected beam path with their edges aligned with the center of the screw holes. Proper reflection alignment was confirmed when the beam passed through the middle of the guards, becoming visible in a third guard placed further down the optical path.

2.2.2 Immersion Chamber as a Positioning Reference

Defining coordinate axes for the movement of the sample is critical for precise positioning. In this system they were defined by using the immersion chamber as a reference.

An image of the stage and chamber system, as well as an illustration of the axis, is seen in Figure 2.7. With this configuration, the illumination plane made by the scanner spans the XZ plane. Since the detection axis is orthogonal to this plane, the acquisition direction is the Y -axis. This configuration justifies the use of the term Y -stack, as volumetric data will be acquired by translating the sample along the Y direction and capturing sequential planes.

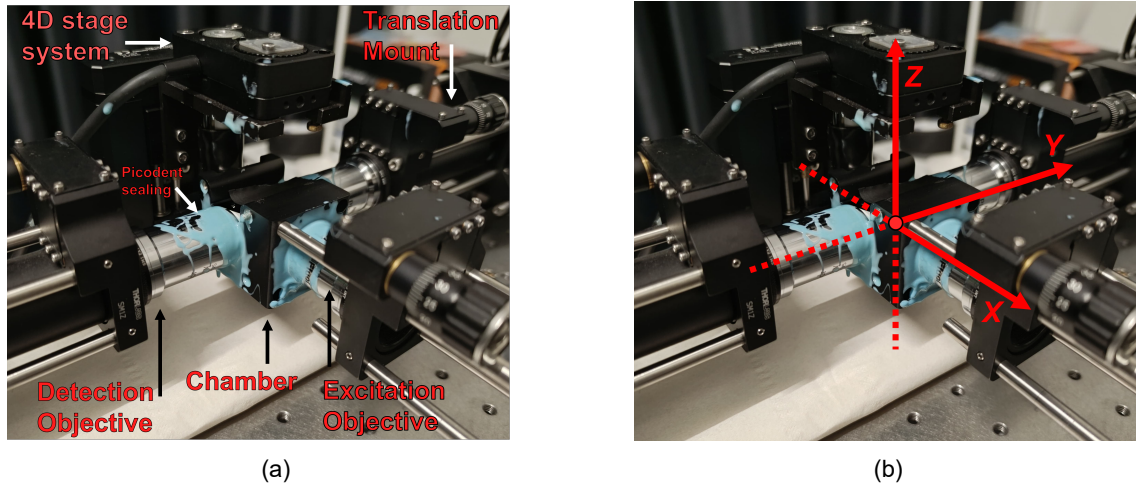


FIGURE 2.7: (a) Image showing the relative position of the illumination and detection objectives to the immersion chamber. (b) Schematic representation of the reference axis of the stage's movement.

2.2.3 Illumination Path

The illumination path system, illustrated in Figure 2.8, is responsible for modulating the collimated laser beam into a dynamically scanned light sheet that selectively illuminates the sample. It begins at the input aperture of the scanner, where the beam enters and is redirected by a pair of orthogonally mounted galvanometric mirrors.

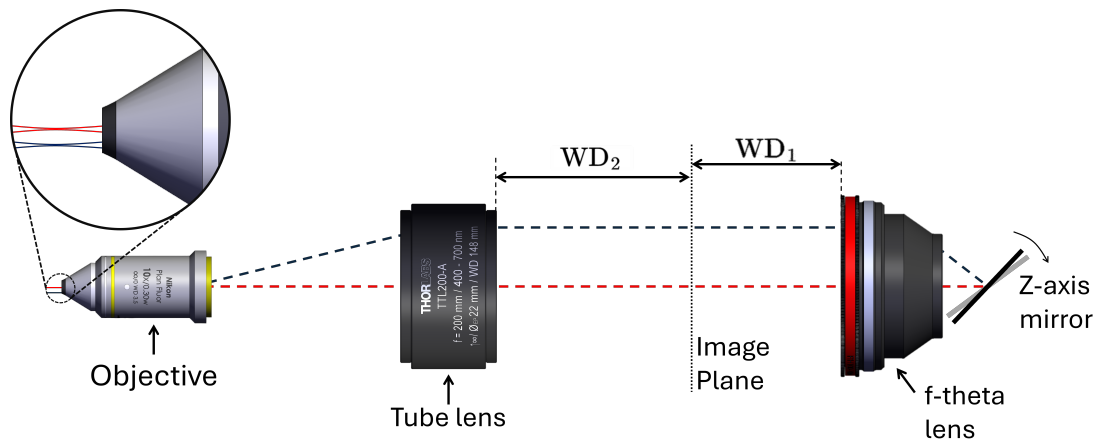


FIGURE 2.8: Illustration of the illumination system's working principle. The movement of Z-mirror results in angular displacements in the input beam, which are then corrected by an f-theta lens. Then a tube lens focuses the incident rays at the back aperture of the objective, forming a thin light sheet at the sample plane.

Each mirror independently controls the beam's angular position along the Y and Z axes. In this setup, only the Z-axis mirror is actuated to laterally scan the beam across the FOV.

In contrast, the Y -axis mirror remains fixed, ensuring beam stability along the orthogonal axis.

The beam exits the scanner through its output aperture and is directed into an f-theta Ronar 160 mm/532 nm lens, which converts the angular displacement of the beam from the Z -axis mirror into a linear displacement at the lens's image plane at a distance WD_1 (working distance of the f-theta lens). The beam is then passed through a tube lens at a distance WD_2 (working distance of the tube lens) from the image plane, which refocuses the collimated light onto the back aperture of an infinity-corrected immersion objective.

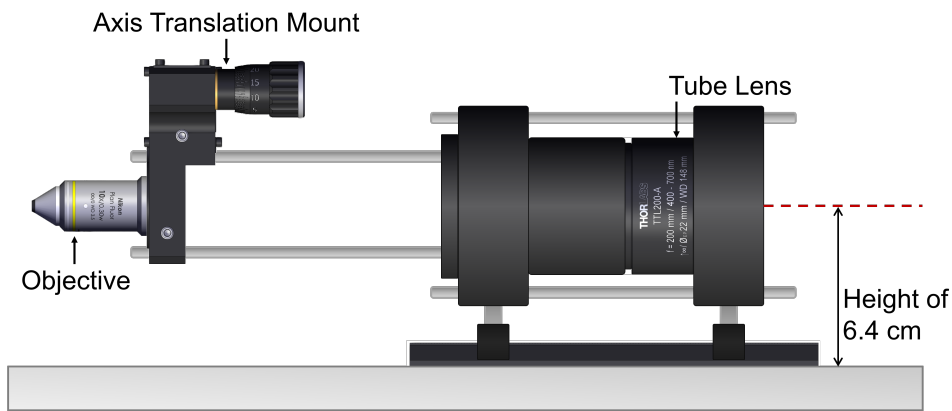


FIGURE 2.9: Illustration of the optical cage system. The objective sits on a translation mount for centering the Gaussian beam in the camera's FOV. The whole setup is mounted for stability and is placed on an optical rail to ease alignment during microscope assembly.

The tube lens and objective are coupled in a 16 mm optical cage system to provide stability. The objective itself is mounted on top of a translation optic mount, used to center the Gaussian beam in the camera's FOV. This entire system sits on top of an optical rail, which facilitates alignment and adjustments during assembly procedures when performed on the microscope. The optical cage system is illustrated in Figure 2.9.

The optical axis of the tube lens, when sitting on top of the rail, is at a height of 6.4 cm above the optical table. As the scanner's original mechanical design does not match this height, a custom-machined scanner holder is used to elevate it, as illustrated in Figure 2.10. This ensures the height of the scanner's aperture matches the tube lens's optical axis. A 3D-printed camera holder also elevates the center of the camera's sensor to that same height. Simultaneously, it is for this reason that the scanner's input aperture is located at a height of 7.4 cm.

The Nikon CFI Plan Fluorite $10\times/0.3\text{NA}$ immersion objective that is used is a water-based dipping lens and is connected to a custom sample chamber designed with three orthogonally arranged objective entrances, which are sealed with Picodent [54], as is shown in Figure 2.7a. For imaging, the chamber's internal volume is filled with water, allowing the sample to be immersed and enabling one-sided illumination and dual-axis fluorescence detection.

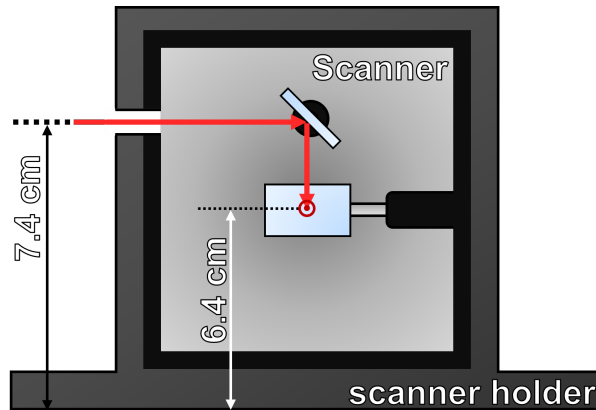


FIGURE 2.10: Illustration of the scanner holder, which elevates the scanner and output beam to match the optical cage system's height of 6.4 cm.

2.2.4 Image Acquisition

The image acquisition system is responsible for detecting the fluorescence emitted by the sample when it is excited by the scanned light sheet. The system shares the illumination path's configuration, but its logic is reversed.

The detection path follows a standard infinity-corrected optical design. Starting at the objective, it collects the fluorescence light emitted by the illumination focal plane and transforms every point into a collimated beam with a specific angular direction. It then propagates towards the tube lens, which focuses it back into an image plane. For this reason, the camera's sensor is placed at the image plane defined by the WD, maximizing the image's focus and resolution.

Between the tube lens and the camera's sensor, the filter wheel is placed to insert filters into the optical path, allowing only the emitted fluorescence to be transmitted and detected by the camera.

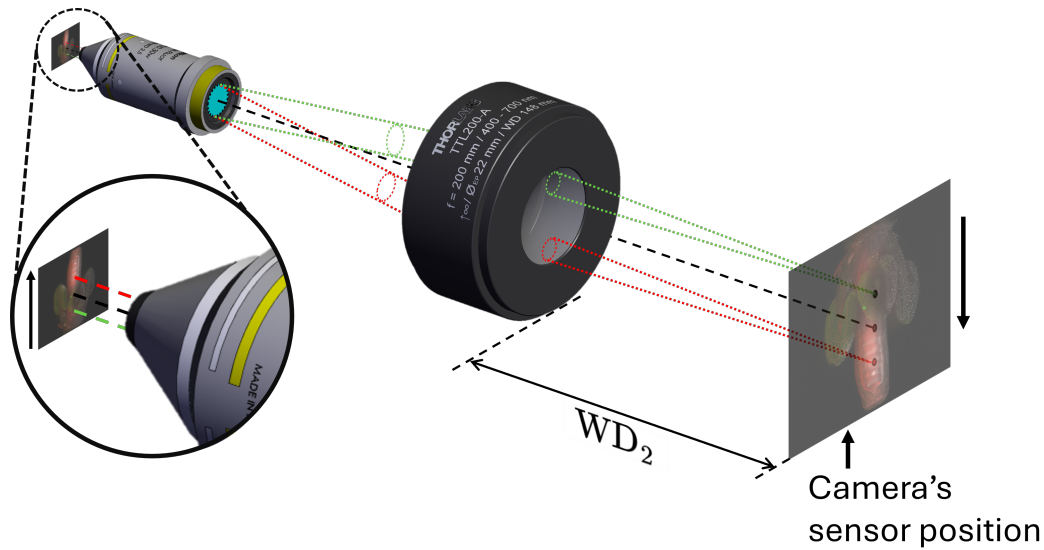


FIGURE 2.11: Image formation in the infinity-corrected objective and tube lens system. Fluorescent light from the sample at the focal plane of the objective is collected and converted into collimated rays, each with a different angular direction. They are then refocused by the tube lens to form a magnified image at its image plane, where the camera sensor is positioned. The black dashed line represents the optical axis of the system, while the red and green rays illustrate how angularly distinct rays from the sample are steered by the lens to specific positions on the image.

Like in the illumination path system, the objective and tube lens are mounted on a 16 mm optical stage system. It is also placed on top of an optical rail to simplify adjustments during the microscope's assembly. The objective is also connected to an axis translation mount to allow focusing of the image and centering the fluorescence emission in the FOV.

A schematic of this detection pathway is shown in Figure 2.11, illustrating the transformation of the fluorescence signal into collimated light by the objective and the image formation at the camera's sensor by the tube lens.

In terms of spatial resolution, the image acquisition system achieves cellular-level detail, with a validated pixel size of $0.66 \mu\text{m}$. This validation was performed by imaging a specific feature within a zebrafish sample and recording its precise position according to the stage coordinates at each corner of the camera's field of view. When compared to the camera's theoretical pixel size of $0.65 \mu\text{m}$ [52], accounting for the $10\times$ reduction introduced by the objective, the validated measurement closely matches the expected value, confirming the system's spatial calibration accuracy.

2.3 Control & Coordination

The successful operation of the microscope depends not only on the precise optical alignment of its components but also on the management of their functionalities during image acquisition. Synchronization is achieved through a coordinated control strategy in which a single master controller governs the behavior of multiple hardware elements: the scanner, the lasers, and the cameras. The objective is to ensure precise temporal coordination between illumination, scanning, and detection in a manner that maximizes image quality, velocity in image acquisition, and experimental reproducibility.

In this section, a description of the electronic connections that were developed to properly coordinate the system is presented.

2.3.1 Master Control via RTC5 and Synchronization Strategy

At the core of this coordination is the ScanLab RTC5 control board, which is an interface developed primarily for industrial laser engraving applications [45]. Despite its original intent, its capability to simultaneously drive the galvanometric mirrors and generate digital/analog output signals makes it particularly suitable for microscopy applications.

The initiation of the “marking” procedure, which in reality corresponds to the simple scanning operation, involves one of the pins on the “LASER” connection on the board issuing a digital “LASERON” signal [45]. In its default application, the signal triggers the laser’s emission for engraving. However, in this context, we utilized it as an external trigger for the Hamamatsu cameras. At the same time, to enhance image quality, a global exposure setting is used on the cameras, in which all the pixels start exposing at the signal’s activation and stop exposing at the signal’s deactivation.

Simultaneously, the RTC5 offers two digital outputs (DIGITAL OUT1/2) and two analog outputs (ANALOG OUT1/2) on the pins of that same LASER connection [45]. These can be programmatically controlled and can be leveraged by specifically routing each to the digital modulation inputs of the four Coherent LX/LS lasers.

This system properly connects the microscope electronically, enabling synchronized multi-channel imaging and a centralized logic system in which all devices respond to a unified set of timing commands. The result is a streamlined acquisition workflow that minimizes temporal drift between components.

2.3.2 Hardware Development for Signal Distribution

To enable the physical connection of the RTC5 board to all lasers and cameras, each having distinct electrical requirements, a dedicated communications box was developed. This intermediary interface facilitates voltage level conversion, impedance matching, and cable management, ensuring reliable signal propagation throughout the system.

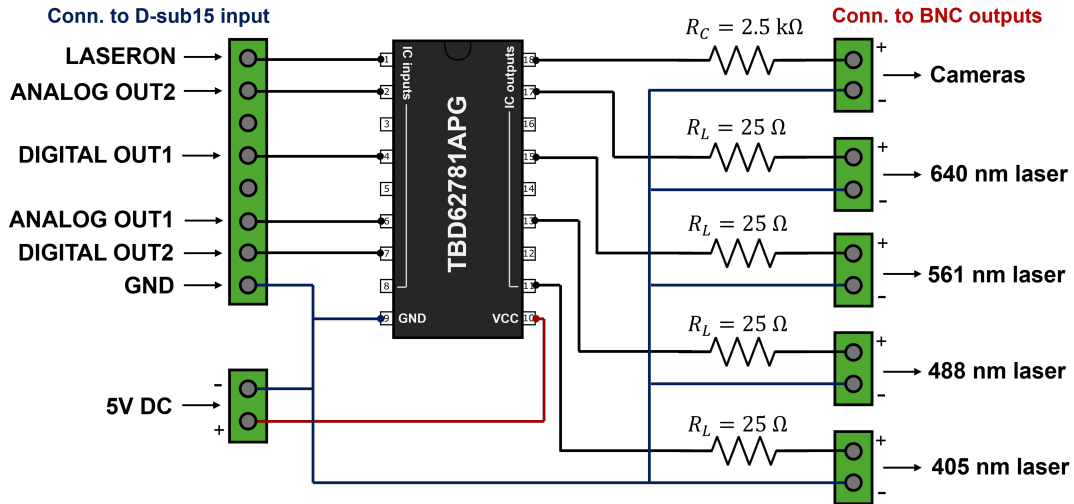


FIGURE 2.12: Schematic of the developed circuit. On the left the terminal breakout connections receive signals from the "LASER" connection of the RTC5 board. Notice the connection from the "LASER" ground to the 5 V DC negative terminal, which connects to the GND connection of the IC and thus correctly references all its inputs and outputs. On the right, the outputs are connected to BNC terminals attached to the box.

A schematic of the developed circuit can be seen in Figure 2.12. The communication box receives signals from the LASER connection of the RTC5 via a D-Sub 15 male/female (M/F) cable, which connects to a D-Sub 15 terminal breakout board inside the box. From this breakout, all digital and analog signals are routed to a custom-built circuit based on the TB62781APG integrated circuit (IC), which utilizes a Darlington transistor array to amplify control signals [55]. The IC is powered by a 5 V DC supply, providing up to 2 A of current and sufficient power to enable the activation of the lasers and cameras. This connection is made by an input DC terminal on the communications box that receives power directly from the grid.

Since the lasers and cameras expect TTL-level signals at different impedances ($50\ \Omega$ for each laser and $10\ \text{k}\Omega$ for the cameras), voltage-dividing resistors are included after each of the five active outputs to step down the 5 V signal to the required 3.3 V logic level. For each of the four laser lines, a $25\ \Omega$ resistor is applied. The cameras share the same output

signal line. Therefore, they exhibit an effective impedance of $5\text{ k}\Omega$. For this reason, a $2.5\text{ k}\Omega$ series resistor is used.

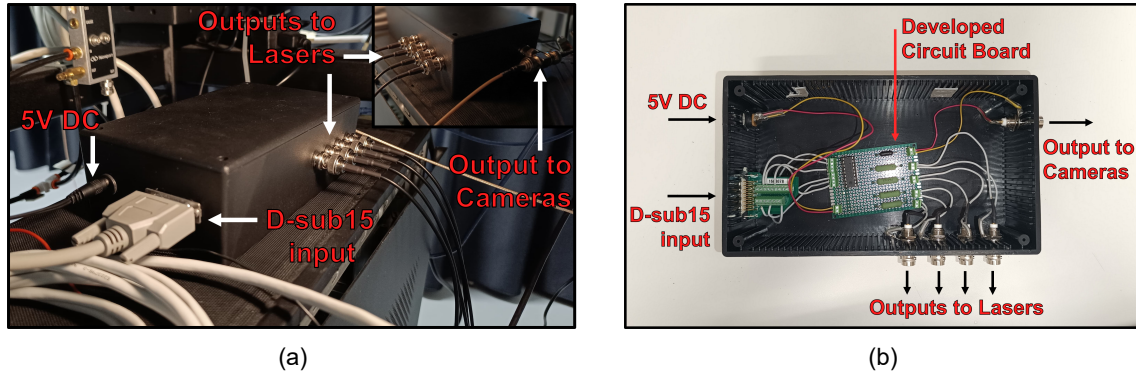


FIGURE 2.13: (a) Image showing the communications box located on top of the control computer, displaying all input and output connections. (b) Image displaying the inside of the communications box and how the connections from the input and output connectors to the circuit board are made.

For the effective physical connections on the box, five BNC output terminals were installed, which are linked to the transistor circuit. Of these, four BNC connectors are dedicated to triggering the lasers. Each of them connects to a Coherent OBIS LX/LS device with a BNC-SMB M/M coaxial cable, ensuring signal integrity and electromagnetic shielding. The fifth BNC connector is used to distribute the synchronized trigger signal to both Hamamatsu cameras. A passive BNC Y-splitter is used, which bifurcates the output signal into two BNC-SMA M/M cables, each routed to the external trigger input of a camera. The constructed box is shown in Figure 2.13.

The use of the RTC5 as a single master brings significant advantages in synchronizing the microscope's key components, ensuring that exposure, illumination, and scanning are perfectly aligned in time. The tight synchronization also enables different types of acquisition strategies. The system supports both the Y - λ order, where a full stack is captured with one laser before moving to the next, and the λ - Y order, where all lasers are fired sequentially at each Y -plane. Switching between these modes requires only a change in the used acquisition algorithm, without modifying the hardware connections.

Finally, the communications box was verified, having achieved the intended behavior. For the verification, the input and output signals were measured using an oscilloscope. The input beams behaved as described in the RTC5 manual, correctly activating the Darlington transistor and routing the DC voltage to the appropriate BNC output. When the input signal was active, a 3.3 V signal was measured across all BNC outputs when connected to their

respective devices, as was intended. Additionally, a 0 V signal was measured when the transistor was not being activated. This technical achievement also manifested in the lasers and cameras, which correctly responded to the emitted signals.

3 Software Development

The construction of a fully functional LSFM requires not only the physical integration of high-precision optical and electromechanical components but also a robust software layer that enables them to operate in a flexible, user-friendly, and reproducible manner. In this project, the software component was designed and implemented entirely in-house to provide complete control over all subsystems of the microscope while ensuring an intuitive user interaction.

In this chapter, the necessity of in-house implementation and the broad implications of chosen software tools and architectures are discussed. Then, the software implementation is detailed.

3.1 Design Requirements and Tools

The primary objectives for the software were defined at the start of development to address the following key requirements:

1. Control over all hardware devices.
2. Real-time visualization of the sample and snapshot capture.
3. Y-stack functionality and time-lapse acquisition with any choice of lasers and cameras.
4. Creation of a text file containing all relevant acquisition information, such as positioning and each device's settings.
5. An intuitive GUI to facilitate acquisition workflows for the users.

The decision not to adopt pre-existing microscope control software (commercial or open-source) was a direct response to the system's unique hardware configuration. Off-the-shelf solutions, such as μ Manager or Cockpit, offer general-purpose frameworks capable of supporting a diverse range of devices [35, 37]. However, they inherently prioritize broad compatibility over fine-tuned performance.

Cockpit, for example, offers a GUI with widgets, device abstraction, and image visualization. While its design does overlap with some goals of this software, it does not support the

SCANcube 7 scanner control through the use of the python-microscope library to establish communication [56]. Furthermore, it poses operational constraints since it is optimized for typical SPIM configurations. Consequently, implementing it in the ALM microscope would have required considerable adaptation, likely outweighing the benefits of reusing code.

In contrast, the ALM system is highly customizable. No available platform provides the specificity of control necessary to match the system's coordination demands, particularly when considering the tight synchronization of laser digital modulation, scanning, and camera external triggering. For all these reasons, the decision was made to build totally custom software that would be tailored for our specific use case.

3.1.1 Python as the Programming Language

Python was selected as the programming language due to its ease of use and extensive scientific ecosystem [57]. Python's extensive library in scientific computing, real-time communication (e.g., pyVISA, PIPython, ctypes), and GUI construction (e.g., PySide6) make it an optimal tool for the development of this type of system.

While there were other options such as C++, C#, or LabVIEW, each came with drawbacks. C++ offers high performance but significantly greater development complexity and limited GUI flexibility [58]. C# has the available Windows Presentation Foundation (WPF) GUI framework but suffers from a weaker community adoption in microscopy software development [59]. Finally, LabVIEW excels in hardware interfacing but is not suited for threaded acquisition logic and does not follow a text-based structure of general programming languages.

In contrast, Python not only supports high-level abstraction but also offers long-term maintainability due to its community-driven evolution and widespread academic adoption. It is highly probable that Python will remain a cornerstone of scientific programming in the coming decade.

3.1.2 Integration of napari

Napari is an open-source image viewer developed in Python, designed for high-dimensional microscopy data [60]. Its choice as the software's image viewer tool was based on its support for live image display and management of multiple camera data through layers.

Alternative options, such as matplotlib or OpenGL rendering, would respectively lack interactive responsiveness or require considerable complexity. Since this system needs real-time image rendering, any other implementation would not be as optimal of a fit and instead be detrimental, since rapid image rendering is essential during alignment and acquisition.

Furthermore, napari is compatible with PySide6, used to construct the software's GUI, and can easily read image data that follows the OME-NGFF specification through the OME-Zarr format. This was also the chosen format for the generated image files, as will be detailed in section 3.1.3, making napari the optimal choice.

3.1.3 OME-Zarr for Image Storage

The use of OME-Zarr as the format for image storage was influenced by a practical and futuristic outlook [61–63]. The ALM microscope is intended to generate high-resolution, multi-dimensional datasets with structured metadata, and the output must be compatible with modern visualization and analysis tools.

OME-Zarr is a scalable, chunked, and cloud-compatible format developed under the Open Microscopy Environment (OME) initiative. It allows efficient access to individual image slices or ROIs, which is of great importance when working with large volumetric datasets. Furthermore, the format embeds metadata, maintaining acquisition context, which is accessible, for example, in Fiji - a widely used software [64], including by ALM users.

Other alternatives were considered, such as TIFF or OME-TIFF [65, 66], however, they are inefficient in individual slice access and large file sizes. The only drawback of OME-Zarr is its relatively new and developing ecosystem, which is smaller and has fewer tools compared to more established formats. However, the rapid adoption of this standard by the microscopy community makes it a robust and future-proof choice.

3.2 Software Architecture

The software's architecture was conceived around the key principles listed in section 3.1. Figure 3.1 summarizes the full software design, divided into three key components: the back-end communication layer, the control logic layer, and the front-end GUI.

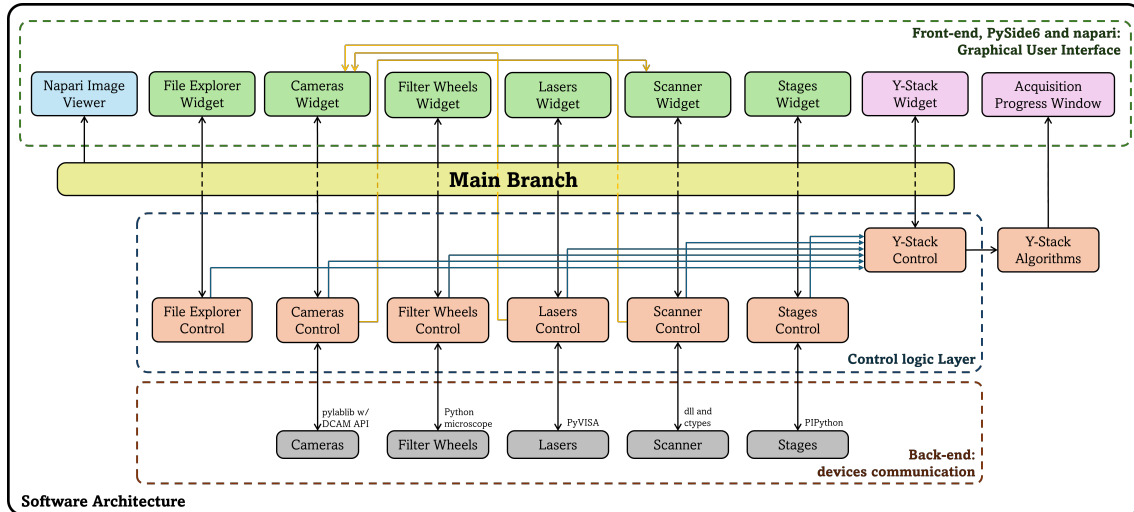


FIGURE 3.1: Illustration of the software's architecture, divided into the back-end, the control logic layer, and the front-end GUI, which enables the user to interact with the system.

At the lowest level, each hardware device is abstracted into an independent Python object responsible for its communication protocol and control. These objects are initialized and maintained within a centralized module called the Main Branch, which serves as the primary communication channel for all necessary purposes. This strategy simplifies all necessary communication between devices and sets a solid foundation for future development.

The front-end, constructed entirely using PySide6, consists of independent widgets dedicated to each device or specific functionalities, such as the File Manager Widget and the Y-Stack Acquisition Widget.

Each device object is connected to a corresponding control class that handles its specific operations and parameters. These control classes are designed to provide clear and isolated interfaces for each device, ensuring a consistent and reliable user experience. For the front-end, there are corresponding GUI widgets that establish direct communication with their associated control classes, allowing the user to send commands and retrieve status updates as needed. Widgets also serve a crucial purpose in limiting the available inputs to each device, thereby ensuring communication without incorrect commands.

Furthermore, some widgets also have the feature of receiving information from other device controllers, in addition to their respective device, a feature that will be described in detail in Section 3.3.

In parallel, image visualization is handled by a napari viewer instance embedded into the

application window. Images from the live camera feeds are directly linked to napari using dedicated threads that handle buffer updates.

3.3 Implementation

The practical implementation of the software in Python followed the architecture outlined in section 3.2. Each widget is built as an isolated yet intercommunicating module, integrated in a central Main Branch that coordinates the devices, all widgets in a GUI layout, and image routing to napari. The remaining sections of the chapter present the design choices and functional capabilities of each component.

Furthermore, to demonstrate these functional capabilities, the control computer's display was recorded during an imaging experiment of a zebrafish larva expressing GFP in the veins. The recording is available on an external resource in Appendix C.2. It presents the whole user workflow, including sample finding, navigation, acquisition parameter definition, and the real-time view of an automated acquisition of a time-lapse at two positions. This workflow is described in more detail in section 3.3.3.2.

Finally, the full software's implementation can be accessed through an external repository found in Appendix C.1.

3.3.1 User Interaction

At startup, the user is first given the option to select the desired folder on the control computer's drive to save all files that are generated during use, with the ability to change this selection after the software's proper initialization.

After this, all physical devices are instantiated as Python objects within the Main Branch, which then distributes them to their respective widgets. When the initialization of the device communication is finished, the full GUI is ready and appears on the display, as is seen in Figure 3.2.

The embedded napari viewer is initialized alongside the GUI, occupying the right section of the main window, while the left side is reserved for the remaining widgets. A background thread continuously monitors camera buffers for new frames and relays them to napari in real time, with each camera's images being routed to different layers in the viewer. This model ensures simultaneous image rendering while maintaining uninterrupted control over all devices.

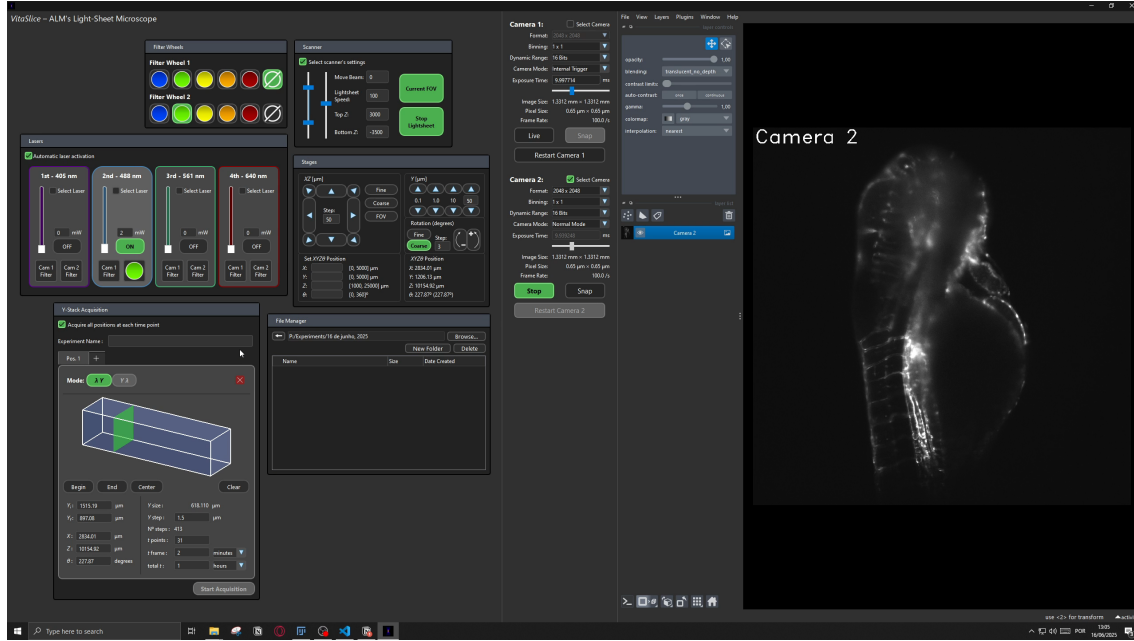


FIGURE 3.2: Main GUI of the custom-developed VitaSlice, showing all control panels and a real-time image of a zebrafish larva. The software provides control over each microscope device, including lasers, filters, scanner, stages, and cameras. On the right, a live rendering of the fluorescent signal acquired by Camera 2 is displayed. As indicated by the control panels, Camera 2 is active, one of the lasers is emitting, and the scanner is generating a light sheet, allowing the sample to be imaged in real time.

Importantly, the GUI seamlessly encapsulates all backend logic, including device communication, synchronization, and data routing. As such, users interact exclusively with interface elements, ensuring safe and robust operation during acquisition workflows.

3.3.2 Individual Device Control Widgets

As mentioned, each device is operated through a dedicated widget, which exposes only the necessary parameters for configuration in a structured and minimal layout, thereby limiting the device's capabilities to what is essential given the microscope's architecture.

All widgets are controlled in parallel threads, ensuring that commands sent to each device do not unnecessarily halt the software.

3.3.2.1 Filter Wheels Widget

This widget provides control over both filter wheels positioned in the emission paths of the image acquisition system. Communication is done with the python-microscope library, which offers a standardized interface to various microscopy devices [56]. It is displayed in Figure 3.3.

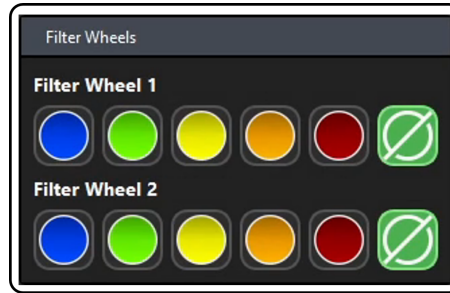


FIGURE 3.3: Filter Wheels Widget of the VitaSlice software. The widget allows users to assign optical filters to the light paths of Camera 1 and 2, respectively. Each color-coded button corresponds to a distinct emission filter, while the crossed circle represents an empty position.

Upon initialization, the widget loads a configuration file that defines the available filters for each wheel. It is a JSON file that includes attributes such as the intended fluorophore to be covered with the filter (as an example to guide the user), spectral bandwidth, slot position, icon file path, and filter condition status. The construction of the GUI on this external file allows future filter modifications or additions without changes to the code.

Each filter wheel has its own space in the GUI, listing the available filters in a row with buttons. Since the filter wheel is always at a given position, one of the buttons is always checked, with both wheels being initialized in an empty filter position. Tooltips display the filter's full name and spectral properties, while the filter icons (from loaded PNG files) provide intuitive visual cues.

This configuration ensures only valid filters can be selected during the microscope's usage. In the case of automated acquisition, the choice of filters occurs in the Laser Widget, as each filter must be associated with the specific laser chosen by the user.

3.3.2.2 Lasers Widget

The laser widget, displayed in Figure 3.4, governs the direct control over the four OBIS lasers used in the microscope (405 nm, 488 nm, 561 nm and 640 nm), each of which is connected via USB to the control computer and accessed through the PyVISA library [67]. Each laser is referred to by its own widget block. A laser power slider enables manual adjustment of output power between 0 and 100 mW, synchronized with a numerical display that also accepts user input. A toggle button is also used to enable or disable emission.

For safety, the logic enforces a power-limiting constraint when turning on a laser: the first time the toggle button is pressed, the system defaults to the minimum power of 1 mW



FIGURE 3.4: Lasers Widget of the VitaSlice software. It allows the user to control four independent lasers, including power adjustments via sliders and on/off toggles. For automated acquisition, each laser must be paired with the appropriate emission filter. The assignment is done separately for each camera with dedicated buttons, which open the filter selection window shown below.

unless it is explicitly overridden by the user. All these power adjustments are immediately sent to the device through a VISA write command, ensuring a near real-time update.

For the definition of laser parameters for the automated acquisition algorithm, the widget features a checkable button ("Select Laser") for each laser, enabling the user to manually select the intended excitation wavelengths. When checked, the selected laser power for automated acquisition is displayed, corresponding to the current emitted power at the time of the button press. Direct numeric input is also enabled by double-clicking the readout label associated with each laser, unlocking more precise tuning for advanced users.

Furthermore, the option to change the order of the lasers on automated acquisition is also available. The default option is to start on the laser with the lowest wavelength (405 nm) and go to the highest. This order minimizes photobleaching, since lower-wavelength photons have higher energy, and it is common for them to excite fluorophores which are the target for longer-wavelength lasers. If they were used as the last emission source, they would excite already previously excited fluorophores with greater energy, promoting photobleaching. While that is the default option, with the dragging-and-dropping of each laser block, the order can be rearranged to the user's preference. The default was chosen with the intent to minimize photobleaching.

Each laser is also associated with the selection of two emission filters for the automated acquisition. The two filters, one per camera, must be manually selected by the user.

The button for each filter opens a window containing images representing the available emission filters. When one is selected, the window closes, and the filter's icon appears on the button.

The chosen settings for automated acquisition are saved in a shared dictionary located in the Main Branch. For this widget, the settings include the selected lasers, their emission power, and the selected filters for each wheel, all presented in list form. During automated acquisition, this dictionary is used to apply consistent laser configurations across all positions and time points.

Finally, there is also available a check button for "Automatic laser activation", which gives the option to the user of the last used laser to automatically start emission after a camera goes "live".

The widget architecture supports extensibility: additional lasers can be added or replace the current ones with minimal changes required to the layout or acquisition routines. This modular design allows the software to be easily adapted for future microscope development.

3.3.2.3 Stages Widget

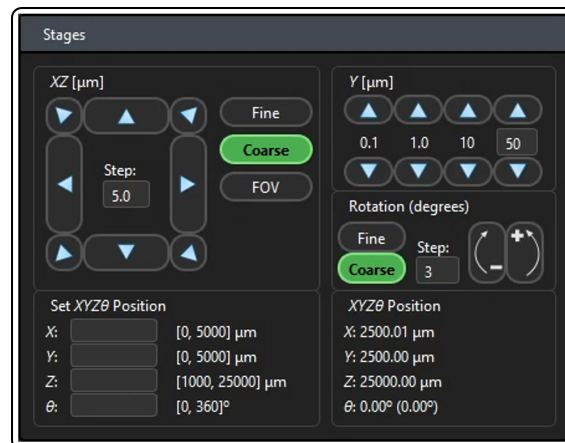


FIGURE 3.5: Stages Widget of the VitaSlice software. The widget provides full control of the motorized stages, which enable precise positioning in the X , Y and θ axes, as well as θ for sample rotation. Additionally, current position coordinates are displayed in real time, and absolute position control is also implemented.

The Stages Widget is responsible for controlling the microscope's motorized positioning system and is displayed in Figure 3.5. Each stage is connected to the C-884 controller,

which communicates with the control computer using the PIPython library [68]. This library interfaces with PI motion controllers and abstracts commands through a high-level command API.

At startup, a referencing routine is automatically triggered, ensuring that each stage axis returns to a known mechanical reference point. Since the stage system is near the sample chamber, collisions are possible. For this reason, the referencing has a fixed order of $Z \rightarrow X \rightarrow Y \rightarrow \theta$, with the Z axis homing at the stage's maximum position, to avoid collisions with the chamber. After the referencing, the X and Y axes go to their middle position to better suit sample placement in the chamber.

The GUI is divided into four control blocks: XZ control, Y -axis control, θ control, and absolute positioning. The X and Z axes are controlled through a two-dimensional joystick emulator composed of virtual arrows, allowing for continuous motion in all directions of the XZ plane. The user can also choose between fine and coarse step sizes through buttons or define an absolute step size in a numeric display.

The θ control consists of two buttons for each angular direction, offering the same fine and coarse movement options, along with a numeric display for selecting a definite step size. The Y axis corresponds to the depth in the real-time camera display. For this reason, its control is performed with various increment options that feature fixed values, each accompanied by up and down buttons. The last option on the right side of the widget offers a user-editable increment value, which is then applied by the up and down buttons.

The widget also includes a “Go To” feature, allowing users to specify target coordinates numerically. The movement of one stage does not halt any possible movement of a different stage, allowing the user to quickly define an absolute ($\theta Z Y X$) position or quickly correct an input done by mistake by entering another command. The stage will follow this last input. Furthermore, since the rotation stage movement is unlimited, the current θ position may not be in the $[0, 360]^\circ$ range. When the user inputs an absolute θ position, the software calculates the closest position to the current one that corresponds to that same angle. The stage moves to that position instead.

A safety feature was also built into the widget: the Z -axis is constrained to prevent it from descending below a minimum threshold, thereby avoiding collision with the chamber. Furthermore, by using an update timer, the absolute positions of all stages are displayed in real-time. The widget frequently queries all stages for position updates, keeping all

information readily available for the user.

3.3.2.4 Scanner Widget

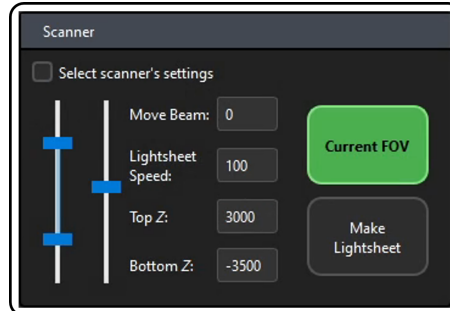


FIGURE 3.6: Scanner Widget of the VitaSlice software. The widget allows users to configure idle beam positioning and scan limits of the Z-axis mirror. "Lightsheet Speed" controls the speed of the scanning movement. A "Current FOV" button detects the current field of view and adapts scan limits accordingly, while a "Make Lightsheet" button commands the continuous scanning movement of the mirror.

The Scanner Widget, displayed in Figure 3.6, controls the Z-mirror of the scanner responsible for the beam scanning that generates the light sheet across the sample. It communicates with the RTC5 board, which drives the scanner, requiring vendor-supplied drivers, dynamic link libraries (DLLs), and correction files to operate correctly. Communication with the board is established via a ctypes interface.

The widget offers a slider-based interface to define the top and bottom scan limits, as well as to control the resting beam position when idle. A toggle button enables or disables the real-time scanning of the beam ("Make Lightsheet" button).

Another key feature of the widget is its ability to synchronize with the camera's FOV. The "Current FOV" button performs an automatic calibration that queries the currently selected camera frame size and maps it to the appropriate sweep amplitude. This ensures that the scanned light sheet fully spans the imaging plane, maximizing fluorescence signal capture while avoiding both overshoot and under-illumination.

The Scanner Widget also connects to the automated acquisition via the shared dictionary. The setting of the parameters happens with the checkable button, and its settings include the current scan bounds and the scan velocity. During automated acquisition, these settings are applied to consistently reproduce the scan behavior across all images.

3.3.2.5 Cameras Widget

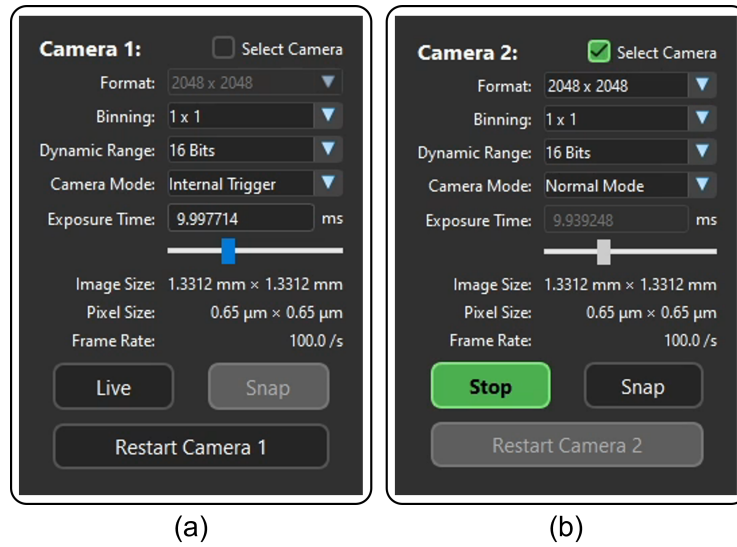


FIGURE 3.7: Camera Widgets of the VitaSlice software. (a) Camera 1 Widget shown in idle mode, with acquisition inactive and the camera not selected for automated acquisition. (b) Camera 2 Widget in "live" mode and selected for automated acquisition. Each widget enables configuration of imaging parameters, including resolution format, binning, bit depth, acquisition mode and exposure time. "Live"/"Stop", "Restart" and "Snap" buttons are also available. Real-time feedback on image size and frame rate are also provided.

There are two similar camera widgets, each responsible for controlling a Hamamatsu camera using the DCAM interface provided through the pyliblib library [69]. The Camera 1 widget in idle mode and the Camera 2 widget in "Live" mode are displayed in Figure 3.7. The user can set the sensor's format, binning configuration, and bit depth (shown as "Dynamic Range" for user familiarity) using drop-down menus. Besides the drop-down menu, the user can also edit the camera's format to the intended format.

These parameters are directly applied to each camera and then validated for the automated acquisition with a checkable button. Exposure time is controlled through both a slider (with a logarithmic scale) and a numeric display, providing fine control for the user over short and long durations. In automated acquisitions, the exposure time setting is not used, as the camera's exposure is directly tied to the scanning of the beam via external triggering (Figure 3.7b). However, the availability of this parameter helps the user in sample-finding workflows.

Three main buttons are available:

- The "Live" mode initiates continuous acquisition via an internal background thread that polls the image buffer to the display.
- "Snap" performs a single-frame acquisition and saves the image.
- "Restart" resets the camera's connection to the control computer, often used when a parameter change unexpectedly shuts down the continuous acquisition.

Although this widget controls the camera's parameters in real-time view and saves the parameters for the automated acquisition, it is not this piece that handles the frame routing to the viewer. Those events occur in a parallel thread in the Main Branch, which will be discussed next, in section 3.3.2.6.

The camera widget plays a central role in synchronizing multi-device operations during triggered acquisition. The "Camera Mode" setting can be applied to "Internal Trigger" or "Normal Mode" (in reference to the to-be-implemented "Lightsheet Mode"), which configures the camera to await external trigger signals.

Additionally, activating "Live" in "Normal Mode" causes the widget to command the Scanner Widget to begin light sheet scanning and simultaneously activates the last laser to emit (as mentioned in section 3.3.2.2). The scanner's TTL outputs then act as external triggers, allowing each scan to fire a frame capture. In this way, simply by activating Live in the camera's external trigger mode, the software automatically receives images from an illuminated sample, making the user experience much more intuitive.

Furthermore, photobleaching mitigation is also implemented. When the "Live" mode is exited in any of the camera's acquisition modes, the widget automatically communicates with the Laser Widget to disable all active lasers. This ensures that fluorescence excitation is strictly limited to imaging intervals.

Additionally, to maintain GUI responsiveness and ensure safe interactions with the user, if continuous acquisition of the camera is ongoing during a parameter change, the widget automatically stops the continuous acquisition, sets the intended parameter, and defines a new continuous acquisition, resuming image display. If not for this implementation, the user would need to stop acquisition, set the parameter, and only then resume the "Live" mode. By comparison, the automatic implementation improves the usability of the software by encouraging the user to explore different parameters to optimize image quality in their experiments.

Finally, for the automated acquisition, the widget also connects to the shared dictionary, which saves the selected format, binning, and bit depth. These parameters are applied to the selected cameras at the beginning of the acquisition, ensuring consistent imaging conditions across all captured frames.

3.3.2.6 Integration of napari and Image Handling

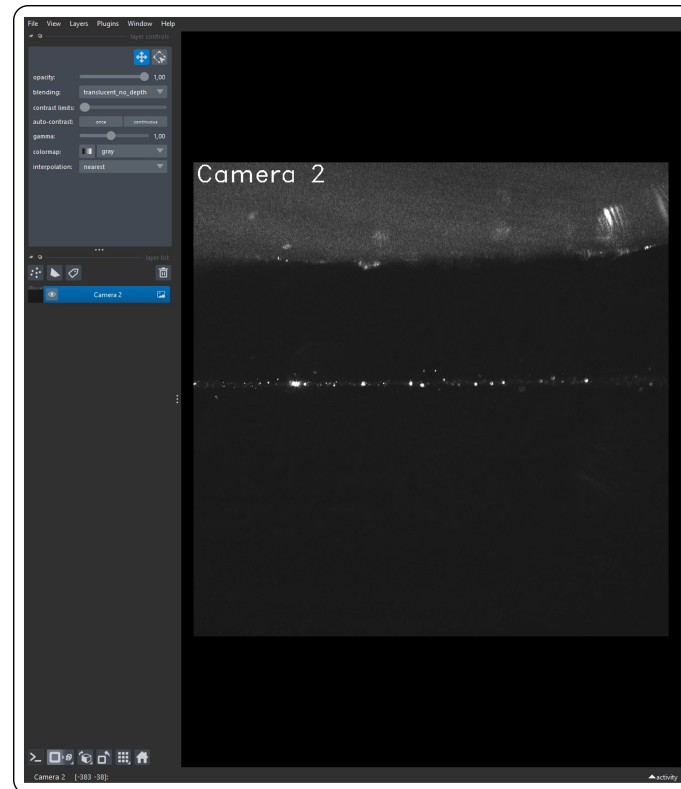


FIGURE 3.8: Right panel of the VitaSlice software, displaying the napari viewer. Camera 2's live feed shows a real-time image of the laser beam and the bottom region of an FEP tube, which contains a sample. The napari viewer provides interactive control over contrast, gamma, and channel visibility.

The napari viewer is embedded on the right side of the software, with the camera's widgets right beside it for accessibility. The right-side display of the software showing the napari viewer can be seen in Figure 3.8. It serves as a live visualization tool, where separate layers are allocated to each camera, enabling simultaneous monitoring of both images. Scroll-based zoom, drag-based panning, and layer-specific display adjustments, such as contrast, LUT, and gamma, are available. Those tools are extremely useful for image visualization, making them indispensable functionalities for the system.

In the Main Branch of the software, there are available slots that receive inputs from each of the camera's widgets when they are set to "Live" or not. When "Live" mode is activated,

a parallel thread is activated that, according to a timer, polls the camera’s buffer for the latest available frame. Using the OpenCV library, that frame is copied, and, depending on the camera, a label that displays its name is added on the frame’s top-left for greater visibility for the user. That frame is then displayed in its appropriate napari layer.

This architecture supports high frame rates without blocking GUI interaction and isolates the display logic from external parameters, thereby enhancing the software’s performance.

3.3.3 Advanced Control: Automated Acquisition and File Management

This section aims to describe the remaining widgets that were developed in addition to the single control for the available devices. They are the Y-Stack Widget, responsible for managing the automated acquisition task, and the File Manager widget, which allows users to view and manage the generated files from snapshots and acquisitions.

3.3.3.1 File Manager Widget

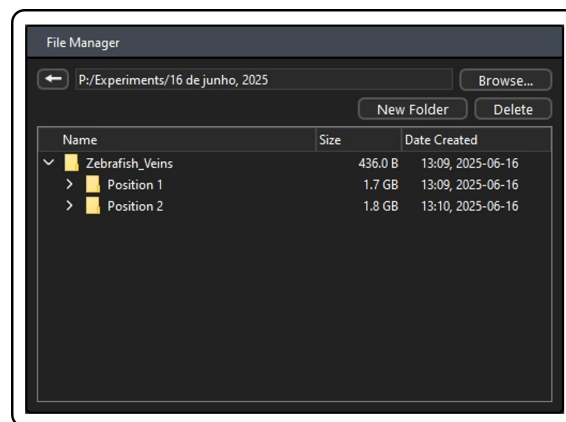


FIGURE 3.9: File Manager Widget of the VitaSlice software. The widget provides navigation in the control computer’s disk and allows management of the acquired datasets. In this example, a multi-position acquisition of a zebrafish larva is stored under the name “Zebrafish_Veins”.

The File Manager Widget, seen in Figure 3.9, offers a self-contained file navigation system directly integrated into the application. Its purpose is to allow users to intuitively browse, select, and manage the generated files during the software’s use. It replicates the features of a standard operating system file manager.

At start-up, the active directory on the widget is the one previously chosen by the user. At the core of the widget is a tree-based directory viewer, allowing users to navigate through

folders in a hierarchical fashion. All directories and files are displayed in vertically stacked rows. On the left side of each row, a triangular toggle icon allows users to expand or collapse a folder. When expanded, its contents appear as indented rows directly beneath it, maintaining a clear parent-child visual structure.

Each item, whether a file or a folder, is represented as a single row with multiple informative columns:

- **Name:** displaying the name of the folder or file, with the correct indentation for its actual path.
- **Size:** displays the file size occupied on the control computer's disk in a readable format, such as "MB" (megabytes) or "GB" (gigabytes), after a background thread calculates it.
- **Creation Date:** displays the creation timestamp of each item, providing an additional informative measure for users to distinguish among acquisition sessions.

Users can click anywhere on a file's row to select it and double-click a folder to enter it as the active directory, updating the path bar at the top of the widget. Alternatively, clicking the button with the left-pointing arrow icon on the toolbar moves the active directory one level up in the folder hierarchy. Finally, the path bar can also be manually edited or updated using the "Browse" button, which opens a native directory selection window.

Moreover, there are more standard folder management actions available:

- **New Folder:** opens a new window to name and create a new directory within the current active directory, which instantly appears on the widget's view.
- **Delete:** removes the selected item after a confirmation window.

The active folder thus serves not just for visualization or navigation, but as the definitive output root for all images and metadata generated by the system. As shown in the schematic of Figure 3.1, the active directory is provided as input for the automated acquisition algorithm.

3.3.3.2 Y-Stack Acquisition Widget

The Y-Stack Widget, displayed in Figure 3.10a, serves as the central interface for configuring and executing volumetric, multi-positional, and time-lapse acquisitions in the microscope. It combines the scan, laser, and camera parameter specifications with an intuitive stage position definition into a unified interface.

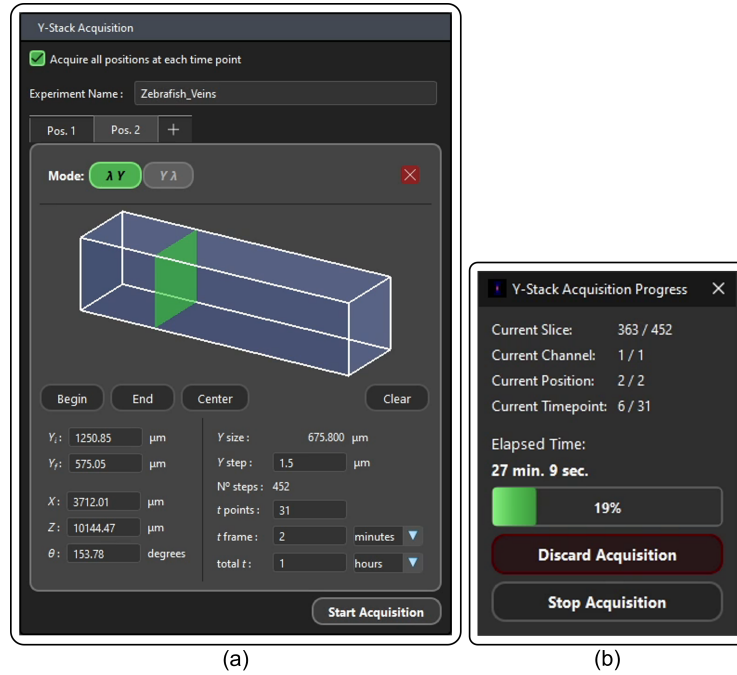


FIGURE 3.10: (a) Y-Stack Acquisition Widget of the VitaSlice software. It enables users to configure volumetric and time-lapse acquisitions by defining volume range, step size, time intervals, time step, and total duration. Multiple positions can be managed within a single experiment by opening new tabs in the widget. (b) Acquisition Progress Window, which provides real-time feedback on the acquisition process. It includes information on the current slice, channel, position, and timepoint, along with elapsed time and completion percentage. Buttons to discard or stop the acquisition are also available.

It has an entry for the name of the experiment, which will be the name of the folder to which the acquisition files will be moved. Additionally, it also displays a dynamic illustration of the microscope's available imaging volume.

It employs a tabulated structure, similar to a web browser, in which the widget assigns one tab per acquisition position where the user can specify the spatial coordinates (X , Y , Z and θ), the sample's depth with an initial and final position (Y_i and Y_f), step size and temporal parameters (number of time points, time interval, and total duration). The (+) button allows the user to dynamically insert new positions, and the (×) button allows their removal.

The spatial specification was designed to align with the real-time sample view. During the workflow:

1. The user navigates towards the sample position that they want to image.
2. The user navigates to one extremity of the sample and presses the "Begin" button. The system captures the current stage positions and displays the Y_i , X , Z , and θ fields.

3. The user then moves to the opposite extremity of the sample and presses “End”, registering the final Y_f coordinate.
4. Upon defining the volume to be imaged, the “Begin” and “End” buttons begin to move the stages to those respective positions. A third button, “Center”, then becomes enabled, responsible for moving the stage to the midpoint between Y_i and Y_f .
5. The widget calculates and displays the total Y -range of the volume as a Y size. The user must then define a step size, allowing the widget to calculate and display the total number of frames to be acquired in the volume.

The number of imaging steps is determined based on the defined step size and on the initial Y_i position. In most cases, the repeated addition of fixed steps to Y_i does not result in an exact match with the final Y_f value. For this reason, the system slightly adjusts the final Y position upwards, beyond the initially recorded Y_f , ensuring that the entire volume is imaged.

For the definition of time-lapses, the user has three available inputs: the number of time-points, the spacing between timepoints, and the total duration of the time-lapse. The system includes a logic that calculates the third missing value from the two inputs the user provides, converting all durations into consistent time units (seconds, minutes, hours, and days). This methodology enables straightforward planning of time-lapse intervals.

An option labeled “Acquire all positions at each time point” toggles between two acquisition orders:

- Checked: This is the default setting, in which all spatial positions defined in the tabs are acquired before moving to the next timepoint. In this option, the time-lapse settings are shared between all positions.
- Unchecked: A complete time-lapse is recorded at the first position before progressing to the next. In this mode, each position accepts different time parameters.

Furthermore, the user can specify a name for the automated acquisition in the “Experiment Name” field above the tab interface. This will then be the name of the output directory where all image and metadata files will be stored. If no name is provided, a default name of “Experiment 1” is used.

If the same name is reused in future acquisitions, the system automatically appends a suffix to avoid overwriting prior data. All output is organized hierarchically, with each

position saved in a sub-directory of this folder (“Position 1”, “Position 2”, etc.). The image files are saved in OME-Zarr format, and a settings file is in text format. The widget also supports two acquisition modes: λY and $Y\lambda$.

This widget communicates with all other control widgets. Before the user can initiate an automated acquisition, a series of parameters must be defined:

- Scanner bounds and speed.
- At least one camera is selected with the respective format, binning, and bit depth.
- At least one laser is selected, along with its corresponding power settings and emission filter for the selected cameras.
- All positional tabs contain valid spatial and temporal parameters. In the case of a simple volumetric acquisition, the default number of time points “1” is assumed.

These parameters are verified via the shared dictionary in the Main Branch. Only when all these conditions are met does the “Start Acquisition” button get enabled. Upon the start of the acquisition, one of the acquisition algorithms begins its execution, and a new window appears to track its progress.

3.3.3.3 Acquisition Progress Window

This window, displayed in Figure 3.10b, provides real-time feedback during automated acquisitions, acting as a centralized status and control interface while these algorithms are running. It is automatically opened at the start of an acquisition and remains visible until it is completed or the user explicitly cancels its operation.

The window is composed of several vertically stacked informative blocks. The current slice index, channel, position, and time point are all displayed in dynamic counters. The order of these fields adapts based on the acquisition mode. When the system is, for example, set to acquire all time points at once for each position, the position counter is displayed after the time point counter, as opposed to what is seen in Figure 3.10. Additionally, if the $Y\lambda$ mode is selected, the current channel appears before the current position display.

These dynamic labels operate by receiving updates from the acquisition algorithm while it functions. These updates are done with Qt signals that send the current and total slice, channel, timepoint, and position at each respective change.

A label displays the elapsed time since the acquisition began, updating every 500 milliseconds. Time is formatted in a readable string (e.g. “1 day, 4 min. 26 sec.”). It serves both as a temporal reference for the user and as an indicator of the system performance throughout time.

Furthermore, a progress bar is displayed, which visually represents the overall acquisition progress as a percentage. It is driven by an image counter that gets incrementally updated. Additionally, the total images are calculated as expressed in Equation 3.1.

$$N_{\text{total slices}} = \sum_{\text{positions}} N_{\text{slices}} \cdot N_{\text{channels}} \cdot N_{\text{time-points}} \quad (3.1)$$

3.3.3.4 Automated Acquisition Algorithms

By nudging the user with the start-up button states on the software, the default acquisition algorithm employed is the “same timepoints for all positions” algorithm executed in λY order. This means that for each time point and position, a complete volumetric stack is acquired using one laser before switching to the next. However, by selecting the acquisition mode and/or the timepoint checkbox on the Y -Stack Acquisition Widget, the other algorithms also become accessible.

The default algorithm’s behavior when compared to the others ensures that the sample is only exposed to one illumination wavelength at a time across the entire sample’s volume, thereby accelerating the acquisition. This algorithm will be detailed in this section.

The acquisition is organized into a multi-level loop structure with the following hierarchy:

1. Timepoint Loop:

The outermost loop iterates over all timepoints. Between timepoints, the system enforces a pause equal to the time interval defined by the user.

2. Position Loop:

For each acquisition tab from the widget, the system moves the linear and rotation stages to the defined $(XYZ\theta)$ coordinates.

3. Laser Loop:

For each selected laser:

- The corresponding power is set, and emission is enabled.

- Filter wheels are rotated to the selected filter for the selected cameras.
- The selected cameras are set to external trigger mode, and a buffer is configured with the number of images corresponding to the entire Y -Stack.
- The system starts the Y -stack across the defined range.

4. Slice Loop:

Within each laser, the stage moves from the initial position Y_i to the final position Y_f , in steps defined by the user. At each step:

- The beam is swept with the Z -mirror of the scanner.
- Trigger pulses are sent to the camera, initially from the RTC5 board during the scan, then routed and amplified through the communications box.
- Each acquired frame is stored in the camera's buffer.

After the entire sample volume is imaged using one laser, the laser is disabled, and the Y -stack dataset is read directly from the buffer into the control computer's disk within an OME-Zarr dataset. The saving in this format will be further detailed in section 3.3.4. After the transfer of the data, acquisition continues to the next laser, position, or time point.

It is important to note that, as previously mentioned, while the acquisition is in progress, continuous information is routed from the algorithm to the "Acquisition Progress" window via Qt signals, allowing for tracking of the current slice, laser, position and, time point. Additionally, as the algorithm tracks this information, it also continuously checks at each stage whether a user input to stop the acquisition has been made. This continuous check happens even when the system is idle during a time interval.

In addition to the default λY mode with shared time points, the system has three alternative acquisition algorithms available, enabling greater flexibility for the user in their required experiments:

- **λY Mode, no shared time points:**

This algorithm allows each position to have its own temporal parameters. Each tab specifies a unique number of timepoints and spacing, enabling heterogeneous acquisition designs across positions. In contrast to the loop previously described, the first loop becomes the position loop, and then the algorithm iterates over timepoints, leaving the rest of the logic unchanged.

• **$Y\lambda$ Mode, shared time points:**

In this configuration, the acquisition order is reversed: for each slice, all lasers are sequentially triggered before the stage advances in the Y direction. This acquisition mode, rather than simply enabling continuous emission for the entire volume, utilizes external modulation triggered by the RTC5 board signals. For every slice:

1. The stage is moved to the appropriate Y -coordinate.
2. The filter wheels change their position to the appropriate filter.
3. The scanner's Z -mirror starts the scanning motion.
4. Two signals are sent from the RTC5 board to the communications box, where they are amplified and routed to the cameras and laser.
5. Due to the signal reception, the laser starts emitting, and the camera is exposed during the scanning motion of the Z -mirror.
6. After the scanning motion has ended, the RTC5 board stops emitting both signals, resulting in the termination of the camera's exposure and the laser's emission. The next frame can now be acquired.

In this acquisition mode, only after all lasers have been cycled for the current Y position does the stage move on to the next.

• **$Y\lambda$ Mode, no shared time points:**

It follows the same methodology that was previously discussed, with each position having its own number of timepoints and time interval. Within each timepoint, all selected lasers are used sequentially before the stage moves to the next Y position.

TABLE 3.1: Comparison between the four acquisition algorithms implemented in the system.

	t-points per position	Y-slice order	Laser order per slice
λY, same t-points	Shared	Full stack per laser	Laser $\rightarrow$ Volume
λY, different t-points	Independent	Full stack per laser	Laser $\rightarrow$ Volume
$Y\lambda$, same t-points	Shared	Slice-by-slice	$Y \rightarrow$ Laser (per slice)
$Y\lambda$, different t-points	Independent	Slice-by-slice	$Y \rightarrow$ Laser (per slice)

The $Y\lambda$ mode, as opposed to the default λY mode, is suitable when the user wants to ensure the precise spatial alignment between different channel images. Finally, all four modes are summarized in Table 3.1.

3.3.4 File Saving and Metadata Recording

Acquired image data are stored using the OME-Zarr format, a scalable, chunked file structure optimized for large-scale, multi-dimensional microscopy data [61]. This format supports efficient downstream processing and is natively compatible with visualization tools such as Fiji and napari. For each position defined by the user, a dataset is written into a dedicated OME-Zarr file, located in a position-specific folder (“Position 1”, “Position 2”, etc.), which is generated in the active directory of the File Manager Widget.

The OME-Zarr datasets generated within each position folder follow an array-like structure, in which image data are stored as multi-dimensional arrays chunked along spatial, channel, and temporal axes. These arrays are accessible in a manner similar to NumPy arrays [70], supporting efficient random access to subsets of the data without requiring the entire dataset to be loaded into memory. Each array is accompanied by metadata files that describe its shape, including its spatial parameters (pixel size, step size, and consequently, voxel size), as well as temporal parameters (time steps and time spacing). The access to readable metadata in a widely used open-source software like Fiji and its ability to store large-scale multi-dimensional data were the main reasons for the choice of the OME-Zarr format.

3.3.4.1 Zarr Array allocation and Frame writing

At the start of each acquisition, before any image is acquired, the software computes the dimensions of the full OME-Zarr array based on the user-defined acquisition parameters:

- Number of timepoints.
- Number of laser channels.
- Number of Y -steps (Y positions).
- Image format corrected for binning.

Using this information, the necessary storage space is allocated directly on the control computer’s disk via a dataset creation method. This step ensures that the full output

array is pre-structured with appropriate chunking and datatype (either `uint8` or `uint16` depending on the chosen bit depth of the camera).

Each camera has its own OME-Zarr file, with the base array under the key “0”, corresponding to the full resolution level. Although the OME-Zarr file format supports multi-scale pyramids, only the full-resolution level is generated during acquisition for time-optimization concerns during the software’s use. Further downscaling can be computed during post-processing.

The image saving during the automated acquisition is performed after an entire volumetric Y-stack is acquired. The full volumetric set of images is read from the camera’s buffer in batch mode and directly written to the allocated Zarr array at the appropriate array indexes. Additionally, the batched reads are handled in blocks of 250 frames, which optimizes the writing of the images compared to writing the full volumetric stack all at once.

3.3.4.2 Metadata Integration

Once imaging concludes, each OME-Zarr file is augmented with the addition of the metadata, which includes:

- Axis definitions (T, λ, Z, Y, X) with appropriate units.
- Voxel sizes are calculated from physical pixel dimensions and binning.
- Time interval spacing.
- OME-OMERO channel annotations, including color and filter name.

These values are automatically inferred from the acquisition configuration defined by the user and stored in the OME-Zarr file as attributes, ensuring readability by third-party software like Fiji and full OME-NGFF compliance.

3.3.4.3 Settings File

Finally, to conclude the created metadata, the software generates a plain-text file for each position summarizing the full experimental context. The file includes:

- Absolute stage coordinates (X, Y, Z, θ).
- The Y spacing and number of Y steps.
- Number of time points and the time step.

- The lasers used and their output power.
- The emission filter per laser and camera.
- The format, binning, and bit depth of the used cameras.
- Scanner parameters (top and bottom bounds of the Z-mirror and scan speed).

The text file is formatted into sections to enhance clear readability. In this way, the meta-data log provides traceability for each experiment, allowing the users to readily verify acquisition parameters. An example of a settings file generated after an acquisition can be seen in Appendix D.

4 Results - System Capabilities

This chapter presents the experimental validation of the light sheet microscope's developed software and hardware. The imaging capabilities of the system are demonstrated using a range of relevant samples, including microglia and oligodendrocyte cells immunolabeled and encapsulated in hydrogel, as well as zebrafish larvae.

These samples were chosen to reflect various imaging challenges, including three-dimensional reconstruction, multi-angle acquisition, and time-lapse performance, and to showcase the system's flexibility, resolution, and stability.

The chapter begins with a description of sample preparation procedures, followed by an analysis of the acquired images.

4.1 Sample Preparation

Proper sample preparation is a fundamental requirement in LSM, as it directly influences image quality and the preservation of biological integrity during acquisition. Given the configuration of the illumination and detection axes, the mounting protocol must ensure precise orientation and mechanical stability.

A widely adopted technique for mounting samples is embedding them in low-melting-point agarose. This creates a stable, hydrated medium with optical properties compatible with the immersion medium used for imaging. In this work, all samples were prepared using variations of this strategy, with specific adaptations for each specimen.

4.1.1 Hydrogel Preparation

To mount the hydrogel containing microglia and oligodendrocyte cells, the preparation followed an embedding approach suitable for soft, bulky specimens. As the hydrogel had a considerable dimension (around 4 mm in diameter and 1.2 mm in height), it was necessary to cut it into smaller portions. For that, a sterile surgical blade was used, and the smaller part of the hydrogel was then carefully introduced into an FEP (fluorinated ethylene propylene) tube, which was attached to a glass capillary. The FEP tube assembled on the capillary served as a refractive index-compatible container for the sample. As the

immersion medium used for the imaging was water, the FEP tube presents an RI similar to that of water, which minimizes optical aberrations.

Once the sample was positioned on the FEP tube, a small plug of agarose (with approximately 2% concentration) was introduced at the bottom to seal it. The assembly was then placed on a 3D-printed claw that tightly fits the rotation stage, minimizing possible drift during acquisition and increasing stability. The sample is then submerged in the chamber for imaging.

4.1.2 Zebrafish Larvae Preparation

Zebrafish larvae were selected for both volumetric acquisitions and time-lapse experiments. To prepare the samples, the larvae were first anesthetized using tricaine mesylate at a concentration of 35 ml/L. Then, a solution of 1% low-melting-point agarose was prepared in E3 medium supplemented with PTU (1-phenyl 2-thiourea). The solution was melted, and once it cooled just above its gelling point, tricaine was added to this solution at the same concentration of 35 ml/L to keep the larvae anesthetized during the experiment.

Then, individual larvae were drawn into an FEP tube mounted on a glass capillary using a piston. Once the agarose polymerized, the sample was immobilized within the tube. The sample was then mounted on the 3D-printed claw, attached to the rotation stage, and submerged for imaging.

4.2 Image Acquisition Results

4.2.1 Hydrogel Imaging

A hydrogel containing primary microglia and oligodendrocyte cells, encapsulated and immunolabeled, was used to validate the system's capabilities for multi-channel imaging. Cells were stained using two different fluorophores: AlexaFluor 488 tagging Iba-1 antibody (microglial marker) and AlexaFluor 568 tagging MBP (the major protein present in myelinating oligodendrocytes). Excitation was performed sequentially using the 488 nm and the 561 nm lasers, respectively. The corresponding filters were also used.

Two different positions were acquired by rotating the sample with the rotation stage, and their full Y-stack range was acquired. A maximum intensity projection was done of the position that showed a better perspective, displayed in Figure 4.1. In the resulting images,

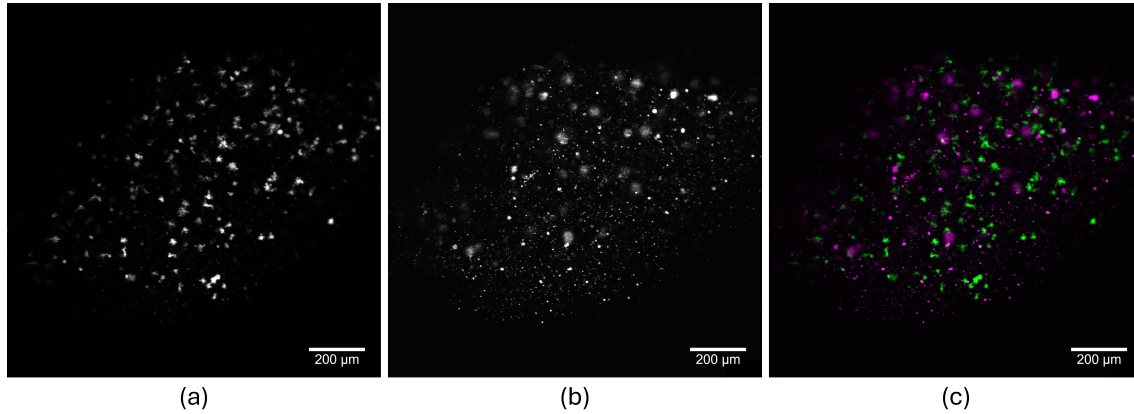


FIGURE 4.1: Representative image of a hydrogel containing primary microglia and oligodendrocyte cells. (a) Primary microglia stained for Iba-1, displaying their typical ramified morphology. (b) Primary oligodendrocytes expressing myelin basic protein (MBP), showing high-intensity signal in both the cell body and ramified structures. (c) Merged image of both channels, with Iba-1 staining shown in green and MBP in magenta.

we can clearly distinguish the two different cell populations, each represented in a different channel, with minimal background signal. These results demonstrate the system's ability to effectively separate fluorescent signals in a dense three-dimensional sample.

Figure 4.1c confirms the accurate spatial localization of both cell populations, without noticeable cross-talk between the channels.

4.2.2 Zebrafish Larvae Imaging

To evaluate the system's performance in imaging a whole organism, two fixed 3-day-old zebrafish larvae were prepared and imaged: a non-infected control and a *Listeria*-infected zebrafish with *Listeria monocytogenes*. The *Listeria monocytogenes* was constitutively expressing a plasmid with an mCherry tag, so all bacteria were fluorescently emitting.

The full Y-stack of both larvae was obtained for two different positions, enabling multi-perspective imaging. A maximum intensity projection from the first perspective is displayed in Figure 4.2. These two images were processed using the same contrast range, with the bacterial clusters seen in Figure 4.2b displaying a greater fluorescent signal.

Additionally, from the second perspective that was acquired, a single slice in both samples is displayed in Figure 4.3. Similarly to the maximum intensity projection, the same channel in each sample was processed with the same contrast range, again displaying the greater fluorescent signal in the infected larva.

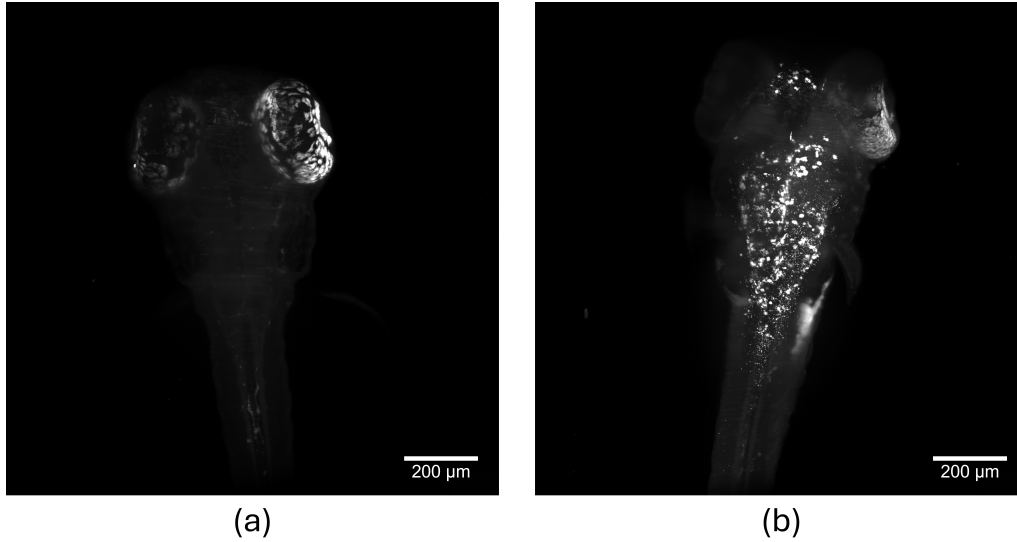


FIGURE 4.2: (a) Maximum intensity projection image of a control zebrafish larva. Only the eye region, which exhibits high autofluorescence, is visible. No other structures are detected. (b) Maximum intensity projection image of an infected zebrafish larva. Dispersed clusters of bacteria are visible along the dorsal region.

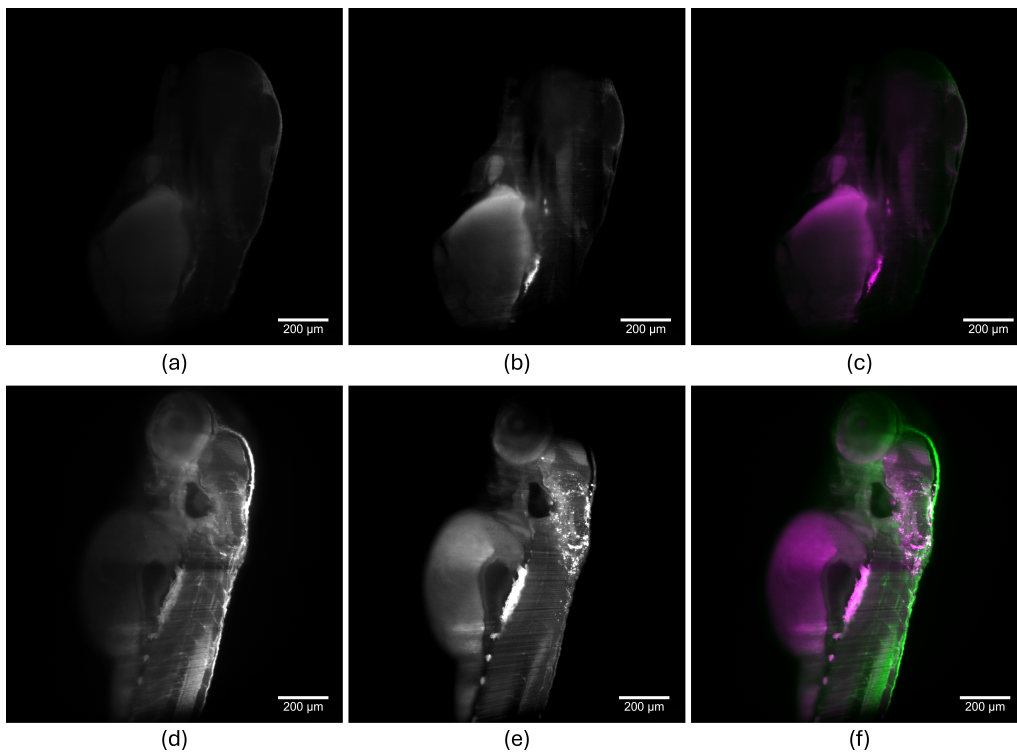


FIGURE 4.3: Zebrafish larvae: control and infected, imaged from a different angle. (a–c) Control zebrafish larvae, showing signal only in the eye region, which exhibits high autofluorescence. Panels (a) and (b) show individual channels acquired using the 488 nm and 561 nm lasers, respectively. Panel (c) displays the merged image, where the 488 nm channel is shown in green and the 561 nm channel in magenta. (d–f) Infected zebrafish larvae, showing clusters of bacteria located over the dorsal region. Panels (d) and (e) show individual channels acquired using the 488 nm and 561 nm lasers, respectively. Panel (f) shows the merged image, with the 488 nm channel in green and the 561 nm channel in magenta.

Excitation was performed using the 488 nm and 561 nm lasers to take advantage of the larvae's autofluorescence and to image the bacteria, respectively. This dual-channel acquisition enabled simultaneous visualization of host structures and microbial distribution within the volume. The control embryo exhibited a weaker signal due to the presence of the simple autofluorescence signal. In contrast, the infected embryo revealed localized fluorescent clusters corresponding to bacterial accumulation sites and a cleaner image from the structural delineation of anatomical features, such as the eye and the brain.

Both samples were imaged across their full Y -stack range using dual-channel illumination. The system successfully acquired high-contrast volumetric data in both channels, enabling the differentiation of host structures and bacterial clusters and confirming their presence in the larva. In this way, the system successfully demonstrated its ability to handle multi-color imaging.

Moreover, each sample was imaged in two distinct angular orientations by rotating the stage. This allowed for the collection of a complementary perspective, thereby enhancing the interpretability of the spatial features visible in the images.

4.2.3 Live Zebrafish Time-lapse

Finally, the system was tested in a time-lapse experiment to assess its ability to perform long-term live imaging. For this purpose, a 24-hour-old zebrafish larva was imaged over 15 hours with a time spacing of 1 hour between images to monitor morphological development. A Y -stack was acquired at each time point, and maximum intensity projections of selected frames are shown in Figure 4.4. Excitation was done using the 488 nm as the larva was expressing constitutively a GFP tag in the veins.

Over the entire experiment, the embryo exhibited clear anatomical changes consistent with normal developmental growth. Notably, the total body width increased significantly, and key structures such as the tail, eyes, and somites became more defined over time. This observable growth confirms the system's ability to capture morphological dynamics over time in live samples.

Additionally, the quality of the data remained consistent across time points and showed no detectable phototoxic effects. The clear reproducibility shown by the acquisition of multiple volumes over time validates the optical stability and confirms its suitability for long-duration imaging experiments.

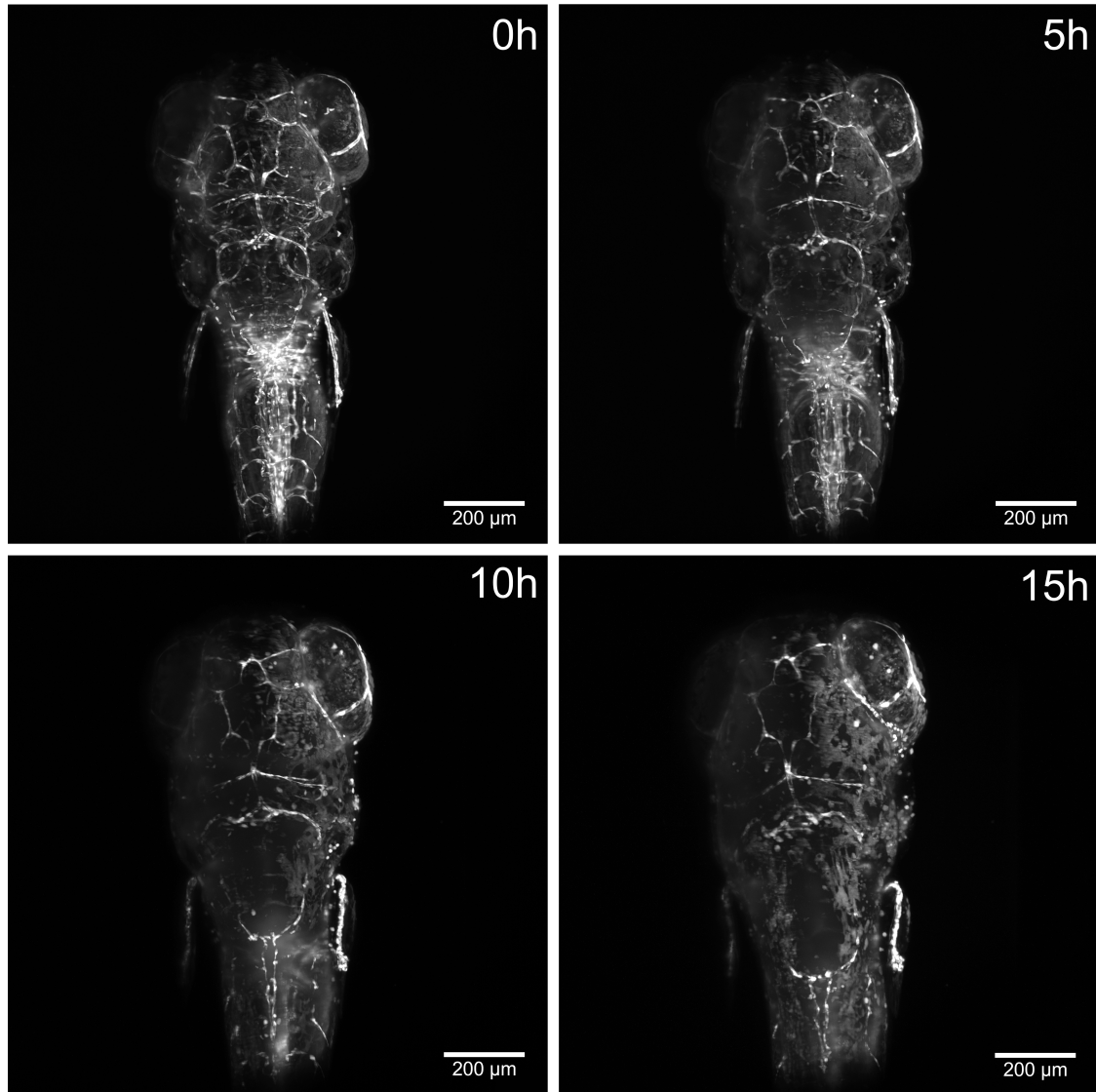


FIGURE 4.4: Time-lapse acquisition of a zebrafish larva. Images were acquired at 1-hour intervals. Shown here are maximum intensity projection images corresponding to timepoints 0h, 5h, 10h, and 15h. The growth of the larva over time is visible, as well as the staining of the veins.

5 Conclusions & Future Work

5.1 Discussion & General Conclusions

The present dissertation has achieved its primary objective: the design and implementation of robust and accessible software to control a custom-built LSFM. The system was developed specifically for operation within ALM, with the goal of bridging the gap between complex instrumentation and intuitive software interaction. From a technical and practical perspective, the results presented validate the approach taken to a fully custom-built software. Additionally, they demonstrate that open and cost-effective alternatives to commercial microscopes are not only viable but also powerful.

The developed system achieved high-frame-rate volumetric imaging, supporting real-time preview, fast post-acquisition visualization, and a storage methodology. Acquisition rates of approximately 470 frames per minute were registered. When accounting for the delays of stage motion, filter motion, and synchronicity of scanner, laser, and a dual camera setup, this image rate is well aligned with the norms for volumetric acquisitions in LSFM. This acquisition rate enabled rapid imaging of both live and fixed samples. In terms of spatial resolution, the system is capable of capturing images at a cellular level of detail, with a validated pixel size of $0.66\text{ }\mu\text{m}$.

A key achievement of the software development is the degree of modularity and extensibility that it enables. The software enables the addition of new laser lines or alteration of filters with minimal software changes. Furthermore, the implementation of OME-Zarr as the primary file format ensures that the software is not only suitable for current needs but also future-proof, given the recent widespread adoption of the OME-NGFF specification in microscopy.

One of the most critical aspects of the system is its suitability for use in a shared research facility, achieved by abstracting the microscope's architectural complexity into an intuitive GUI. Feedback gathered during test sessions with users who provided test samples confirmed the system's accessibility, largely due to its simple appearance and clear, minimal organization.

Compared to commercial systems, such as the Zeiss Lightsheet 7, the developed system offers a significantly more affordable and open solution while retaining essential functionalities, including volumetric and time-lapse acquisition, as well as real-time feedback. While it cannot currently compete with such systems in terms of environmental stability (temperature, CO₂, humidity), this system provides an excellent alternative for laboratories prioritizing flexibility and a more affordable option.

When compared to other custom and academic microscopes, such as OpenSPIM and mesoSPIM, our system stands out positively, since it differentiates itself as being the only one with a practical orientation towards facility use. OpenSPIM, while pioneering as a fully open-source LSFM design, has an interface and architecture that are not optimized for broad, non-expert use. MesoSPIM, on the other hand, also assumes a more engineering-oriented user with the capacity to assemble complex optical systems and custom sample holders, with the purpose of achieving high-resolution images. Thus, our system occupies a distinct position, offering a well-balanced platform that emphasizes usability and ease of deployment.

This project contributes to the effort of making advanced microscopy techniques more accessible and reproducible. By providing full documentation, this work supports a movement of openness in science, specifically in a field that is predominantly dominated by expensive commercial systems.

Finally, it is worth noting that the development of this system coincided with the rapid emergence and evolution of generative artificial intelligence (AI) tools. These have become a clear technological shift and were used in this work to support code writing and technical troubleshooting. This use has ultimately accelerated development and enabled a much more ambitious and polished outcome than what would have been possible in the limited time frame of a master's dissertation. As such, this work also represents this paradigm shift and showcases how the development of complex scientific systems can now be significantly accelerated. In that sense, this work serves as an early indicator of how these new generative AI technologies may enable a new era of rapid scientific innovation across all fields of knowledge.

5.2 Future Work

Despite the system's functional and technical success, there is still considerable room to expand the system's capabilities and refine the already developed system. There are several possible improvements, both in the hardware and software of the system.

Starting with the sample handling process, the system could benefit from modifications to the sample chamber. Currently, the chamber has a very limited available height, which constrains the movement of the capillary and FEP tube in the Z direction. This limitation requires careful sample preparation, as the specimen must be positioned near the start of the capillary/FEP tube for it to be captured by the camera's FOV before the FEP reaches the base of the chamber, leaving the sample out of the image. Additionally, in case we want to image more than one specimen prepared in the same mounting, we face restrictions. Future designs should consider increasing vertical clearance to allow for greater tolerance when preparing samples, as well as enabling the preparation of multiple samples in the same FEP tube.

Furthermore, the current design relies on the optical cage system and the connection to the objectives to maintain its stability. Introducing a dedicated chamber holder would not only improve its mechanical stability but would also facilitate the microscope's assembly when necessary, as well as the chamber mounting process.

Another important ability for the sample chamber to possess would be its thermal regulation, which would enhance the microscope's capacity for live imaging applications. Maintaining at least a constant temperature is of extreme importance during time-lapse acquisitions. One possible solution involves constructing a metallic chamber with built-in machined paths for tubing to pass through, in which temperature-controlled water can circulate. Due to the high thermal conductivity of metals, this would allow a stable temperature equilibrium in the whole chamber volume.

To complete the hardware-related improvements, an upgrade that enables the subsequent implementation of the camera's Light Sheet Mode in software is the replacement of the currently used f-theta lens. While the camera supports acquisition using the rolling shutter mode, its effective implementation depends on the performance of the optical system. The currently employed f-theta lens is a standard model and lacks telecentricity, which means the output beam is not parallel to the optical axis for all input angles.

Consequently, the angular deflections produced by the Z-axis galvanometric mirror do not produce parallel rays, as it is ideally depicted in Figure 2.8. Instead, the non-telecentric tube lens, rather than achieving normal incidence on the tube lens, causes the scanned beam to strike it at an angle, which leads to misalignment at the back aperture of the objective. As a result, at the light sheet plane there are angular deviations present during the laser scanning, which compromises the synchronization between scanning and the rolling shutter exposure.

The solution would then be the employment of a telecentric f-theta lens, which ensures that all output rays remain parallel to the optical axis, regardless of the scan angle. This preserves normal incidence at the tube lens and maintains a consistent beam position at the back aperture of the objective. At the light sheet plane, the synchronization between beam scanning and rolling shutter would then be possible, leading to better optical sectioning and improved image quality. This is particularly important when, due to the thickness of the sample, scattering starts blurring the image.

For the software, a great addition would be the implementation of a robust time-lapse acquisition system that includes features such as the real-time calculation of the estimated acquisition time and accurate tracking of the elapsed time during volume acquisition.

The acquisition of a whole volume still takes a significant amount of time. Consequently, during a whole time-lapse, this extra time starts accumulating, leading to a mismatch between the expected time-lapse time and the real one. The merging of the volume acquisition time frame on the time step in which the system is idle would prevent this issue. In addition to this implementation, an initial computation of the volume's acquisition time could be employed, giving the user the minimum time step that is possible between acquisitions.

A crucial improvement would also be the optimization of the image-saving algorithm. While the current implementation performs adequately, the software would greatly benefit from improvements on the image transfer from the camera's memory to the control computer disk. The current software performs this transfer after each stack, but a solution where the transfer occurs during acquisition via threading would greatly optimize the time efficiency of the system as well as prevent data bottlenecks when dealing with large volumes. In addition to this, providing an estimated file size, calculated from the specified Y-stack settings, would greatly facilitate efficient data management.

Additionally, it would be of great importance to implement a mosaic acquisition function for tiling acquisition with stitching. For samples larger than the camera's FOV, the ability to define the full sample's boundaries and automatically compute a series of adjacent acquisition positions would greatly enhance the system's capabilities. A stitching algorithm could then mount the acquired volumes into a single dataset and vastly improve the system's imaging scalability.

These planned developments are natural and achievable extensions of the work presented in this dissertation. Their implementation would bring the system one step closer to the capabilities of high-end commercial solutions while preserving the flexibility, modularity, and cost-efficiency that only a fully custom-built system can offer.

A Filters available on the micro- scope

The available filters in each filter wheel, along with their target fluorophores and spectral characteristics are listed in Tables A.1 and A.2.

TABLE A.1: Filter configuration in Filter wheel 1.

Wheel Position	Target fluorophore	Transmission range
0	DAPI	525 / 50 nm
1	GFP	525 / 50 nm
2	YFP	545 / 25 nm
3	Alexa 568	610 / 75 nm
4	Alexa 647	700 / 75 nm

TABLE A.2: Configuration of filters in Filter wheel 2.

Wheel Position	Target fluorophore	Transmission range
0	DAPI	452 / 45 nm
1	GFP	525 / 50 nm
2	YFP	537 / 29 nm
3	Alexa 568	610 / 75 nm
4	Alexa 647	710 / 75 nm

B Supplementary Multimedia

B.1 ALM light sheet microscope system photographs

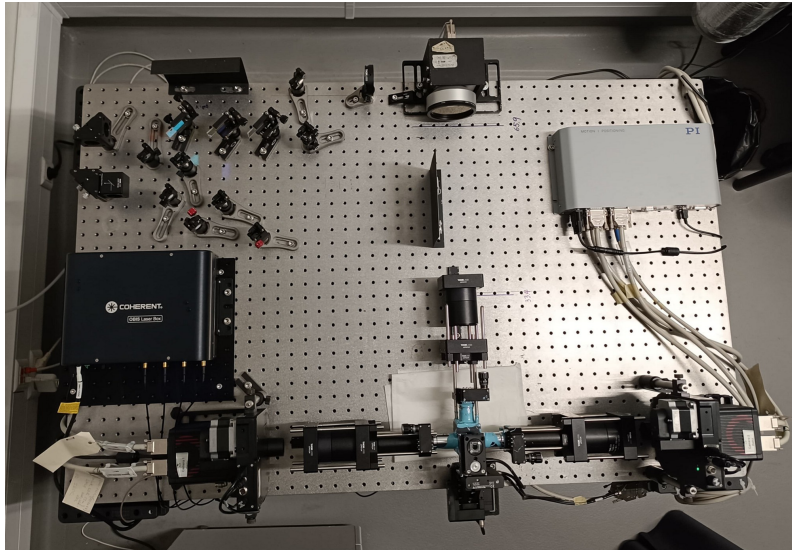


FIGURE B.1: Top view of ALM's light sheet microscope.

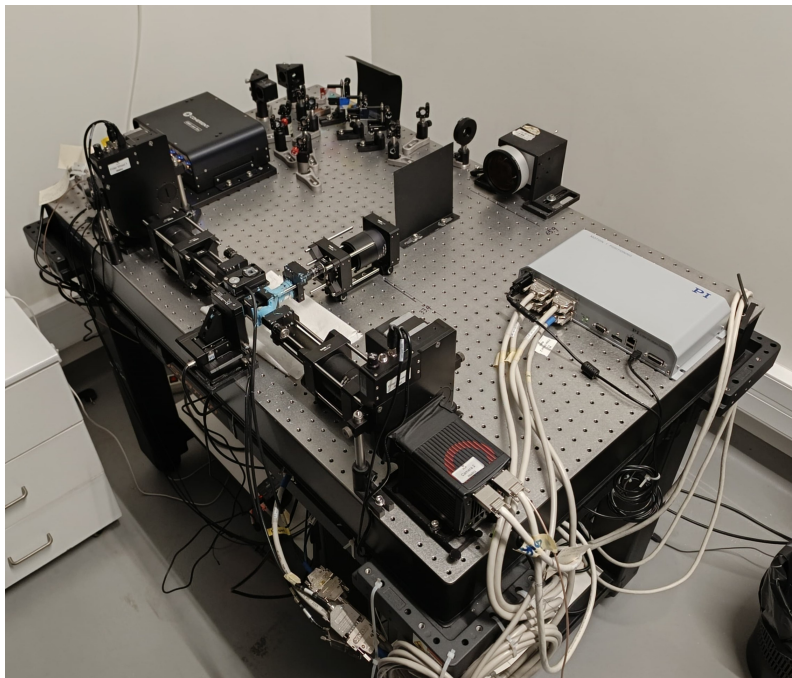
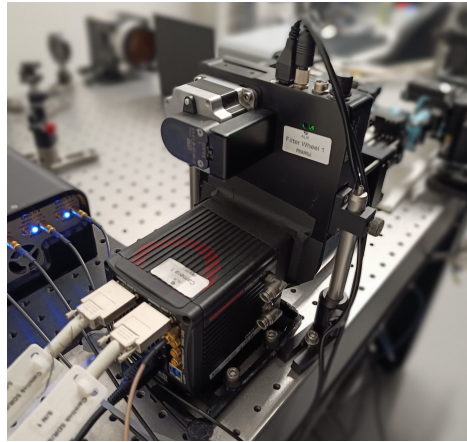


FIGURE B.2: Angled view of ALM's light sheet microscope.

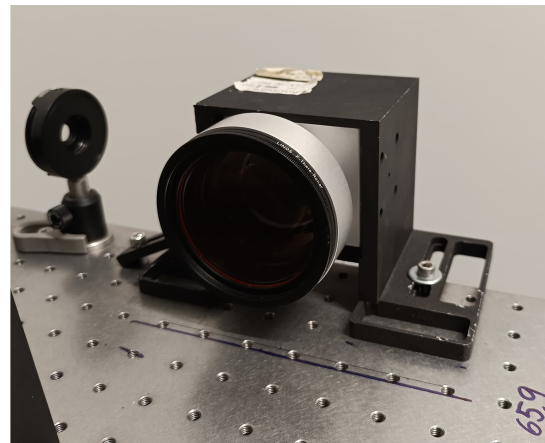
B.2 ALM light sheet microscope device photographs



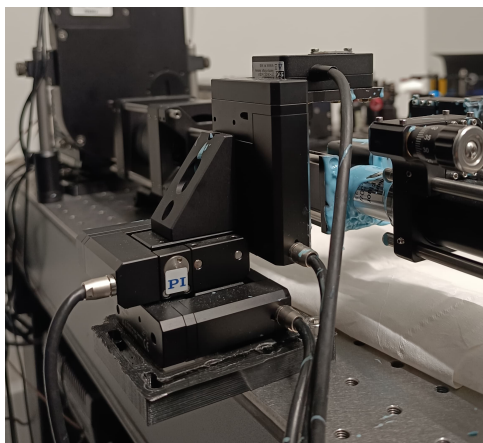
(a)



(b)



(c)



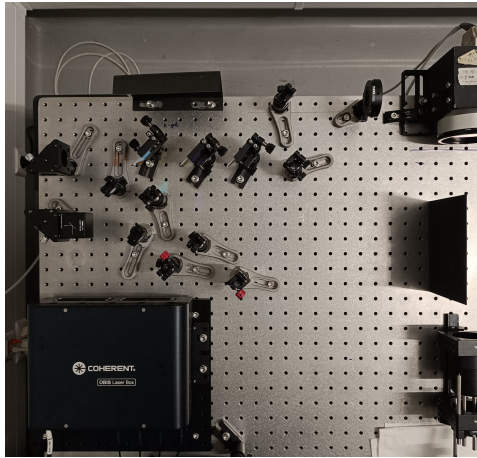
(d)



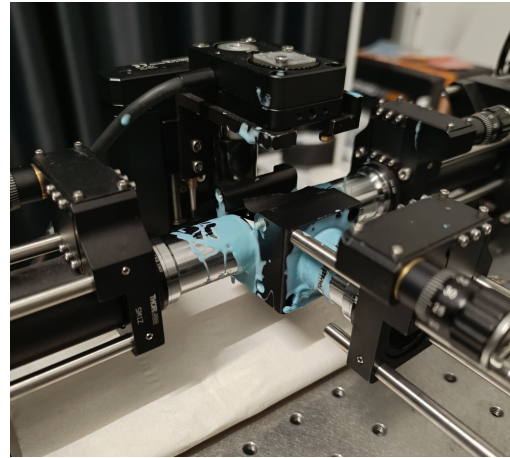
(e)

FIGURE B.3: (a) Camera and filter wheel. (b) Laser box. (c) Scanner. (d) Assembled stage system (e) Stage's motor controller.

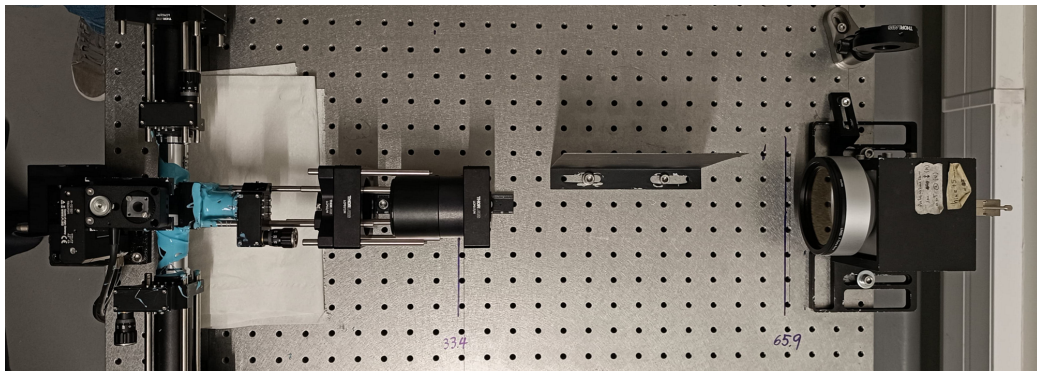
B.3 ALM light sheet microscope subsystem photographs



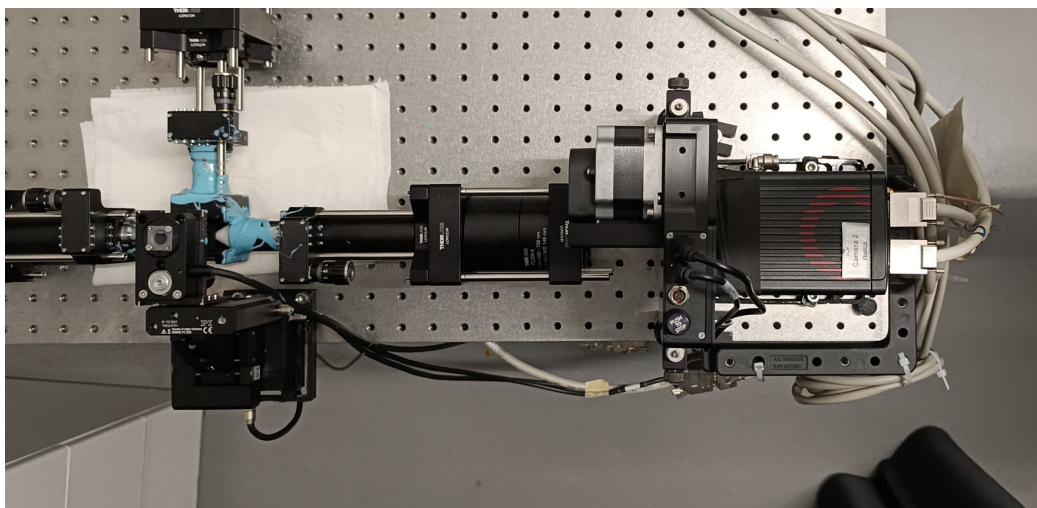
(a)



(b)



(c)



(d)

FIGURE B.4: (a) Reflection optics system. (b) Stage system with sample holder and imaging chamber. (c) Illumination path system. (d) Image acquisition system.

C External Resources

C.1 VitaSlice github repository

The code for the developed software is available at the following repository:

<https://github.com/synkss/VitaSlice>

C.2 Recording of imaging experiment

The display of the control computer was recorded during an image acquisition experiment. The sample is a zebrafish larva, similar to the one presented in section 4.2.3, expressing a GFP tag in the veins.

In the recording, the software launch is presented with the choice of the saving folder and the initialization of communication with the devices. Then, the software main window appears, followed by its use to find the sample. At this first position, the acquisition settings are configured. The sample is then navigated to a second position, which is also included in the time-lapse acquisition. Finally, the time-lapse acquisition starts.

The recording is available in the following domain:

https://www.youtube.com/watch?v=r_R91FeMqz0

D Acquisition settings file example

An example of the text generated in the settings file for the second position of an acquisition experiment is presented:

```
# Experiment Settings:
## Position 2
Acquired 6 times with a time-frame of 2.0 minutes.
-----
### Positional Settings:
X: 3712.010 µm
Z: 10144.470 µm
θ: 153.780 degrees

Initial Y: 1250.850 µm
Final Y: 574.350 µm
Y spacing: 1.500 µm
Nº of steps: 452
-----
### Camera 2 Settings:
Format: 2048 x 2048
Binning: 1 x 1
Dynamic Range: 16 bits
-----
### Scanner Settings:
Speed: 100.0
Top: 3000
Bottom: -3500
-----
### Laser Settings:
#### 1st laser: 488 nm
Laser Power: 2% (2 mW)
Emission Filter 2: GFP (525nm/50)
```

References

- [1] Advanced light microscopy webpage. Institute for Research and Innovation in Health. Accessed on 18 june 2025. [Online]. Available: <https://www.i3s.up.pt/scientific-platform.php?groupid=63> [Cited on page 1.]
- [2] Д.Ильин. Jablonski diagram of fluorescence only. Wikimedia Foundation. Accessed on 18 june 2025. [Online]. Available: https://commons.wikimedia.org/wiki/File:Jablonski_Diagram_of_Fluorescence_Only-en.svg [Cited on page 3.]
- [3] Generic excitation and emission spectra for a fluorescent dye. Chroma Technology Corp. Accessed on 18 june 2025. [Online]. Available: <https://www.chroma.com/support/knowledge-center/about-fluorescence/fluorochrome-spectra> [Cited on page 3.]
- [4] G. G. Stokes, "On the change of refrangibility of light," *Philosophical Transactions of the Royal Society of London*, vol. 142, pp. 463–562, 1852. [Cited on page 3.]
- [5] B. Valeur and M. N. Berberan Santos, "A brief history of fluorescence and phosphorescence before the emergence of quantum theory," *Journal of Chemical Education*, vol. 88, no. 6, pp. 731–738, June 2011. [Cited on page 4.]
- [6] M. Planck, "Zur theorie des gesetzes der energieverteilung im normalspectrum," *Verhandlungen der Deutschen Physikalischen Gesellschaft*, vol. 2, pp. 237–245, 1900. [Cited on page 4.]
- [7] O. Shimomura, F. H. Johnson, and Y. Saiga, "Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan aequorea," *Journal of Cellular and Comparative Physiology*, vol. 59, pp. 223–239, 1962. [Cited on page 4.]
- [8] J. E. Berlier, A. Rothe, G. Buller, J. Bradford, D. R. Gray, B. J. Filanoski, W. G. Telford, S. Yue, J. Liu, C. Cheung, W. Chang, J. D. Hirsch, J. M. Beechem, R. P. Haugland, and R. P. Haugland, "Quantitative comparison of long wavelength alexa fluor dyes to cy dyes: Fluorescence of the dyes and their bioconjugates," *Journal of*

- Histochemistry & Cytochemistry*, vol. 51, no. 12, pp. 1699–1712, Dec. 2003. [Cited on page 4.]
- [9] S. Zaqout, L. L. Becker, and A. M. Kaindl, “Immunofluorescence staining of paraffin sections step by step,” *Frontiers in Neuroanatomy*, vol. 14, p. 582218, 2020. [Cited on page 4.]
- [10] C. Z. M. GmbH, “Principles of fluorescence and fluorescence microscopy,” , Tech. Rep., may 2019, accessed on 19 june 2025. [Online]. Available: https://pages.zeiss.com/rs/896-XMS-794/images/ZEISS-Microscopy_Technology-Note_Principles-of-Fluorescence.pdf [Cited on page 4.]
- [11] R. N. Day and M. W. Davidson, “The fluorescent protein palette: tools for cellular imaging,” *Chemical Society Reviews*, vol. 38, no. 10, pp. 2887–2921, 2009. [Cited on page 4.]
- [12] S. Takahashi, K. Im, S. Mareninov, M. Palma Diaz, and W. Yong, “An introduction to performing immunofluorescence staining,” in *Biobanking*. Springer New York, 2019. [Cited on page 5.]
- [13] Introduction to fluorescence microscopy. Nikon Instruments Inc. Accessed on 19 june 2025. [Online]. Available: <https://www.microscopyu.com/techniques/fluorescence/introduction-to-fluorescence-microscopy> [Cited on page 5.]
- [14] P. J. Shaw, “Bending the rules: Widefield microscopy and the abbe limit of resolution,” *Journal of Cellular Physiology*, vol. 229, no. 1, pp. 132–138, 2014. [Cited on page 5.]
- [15] Introductory confocal concepts. Nikon Instruments Inc. Accessed on 19 june 2025. [Online]. Available: <https://www.microscopyu.com/techniques/confocal/introductory-confocal-conceptsy> [Cited on pages 5 and 6.]
- [16] Multiphoton microscopy. Nikon Instruments Inc. Accessed on 19 june 2025. [Online]. Available: <https://www.microscopyu.com/techniques/multi-photon/multiphoton-microscopy> [Cited on page 6.]
- [17] N. MicroscopyU, “Light sheet fluorescence microscopy,” <https://www.microscopyu.com/techniques/light-sheet/light-sheet-fluorescence-microscopy>, 2025, accessed on 30 june 2025. [Cited on pages 6, 7, 8, 9, and 10.]

- [18] E. H. Stelzer, F. Strobl, B. J. Chang, F. Preusser, S. Preibisch, K. McDole, and R. Fiolka, “Light sheet fluorescence microscopy,” *Nature Reviews Methods Primers*, vol. 1, no. 1, p. 73, 2021. [Cited on pages 6, 7, and 9.]
- [19] R. Hermans, “Gaussian beam waist parameters (derivative of drbob’s original diagram),” <https://commons.wikimedia.org/wiki/File:GaussianBeamWaist.svg>, 2009. [Cited on page 7.]
- [20] M. U. CEITEC – Central European Institute of Technology. Zeiss lightsheet 7 at cellim core facility. . Accessed on 19 june 2025. [Online]. Available: <https://cellim.ceitec.cz/cs/zeiss-lightsheet-7/> [Cited on page 11.]
- [21] M. Optical. Lightsheet 7 imaging system. . Accessed on 19 june 2025. [Online]. Available: <https://micronoptical.com/imaging-systems/lightsheet-7> [Cited on page 11.]
- [22] Z. Microscopy. (2025) Lightsheet 7. . Accessed on 20 June 2025. [Online]. Available: <https://www.zeiss.com/microscopy/en/products/light-microscopes/light-sheet-microscopes/lightsheet-7.html> [Cited on pages 10 and 12.]
- [23] ZEISS Microscopy. (2025) Lattice Lightsheet 7. Accessed on 20 June 2025. [Online]. Available: <https://www.zeiss.com/microscopy/en/products/light-microscopes/light-sheet-microscopes/lattice-lightsheet-7.html> [Cited on page 11.]
- [24] L. Microsystems. Leica stellaris dls. . Accessed on 19 june 2025. [Online]. Available: <https://www.leica-microsystems.com/pt/produtos/microscopios-confocais/p/stellaris-dls/> [Cited on page 12.]
- [25] B. Corporation. (2025) Muvi spim family – light sheet microscopes. . Accessed on 20 June 2025. [Online]. Available: <https://www.bruker.com/en/products-and-solutions/fluorescence-microscopy/light-sheet-microscopes/muvi-spim-family.html>
- [26] L. Microsystems, “Viventis deep dual view light sheet fluorescence microscope,” 2025, accessed on 30 june 2025. [Online]. Available: <https://www.leica-microsystems.com/products/p/viventis-deep/specification/> [Cited on page 12.]
- [27] O. community. Openspim. OpenSPIM. Accessed on 19 june 2025. [Online]. Available: https://openspim.org/Who_has_an_OpenSPIM [Cited on page 13.]

- [28] mesoSPIM Initiative. Existing mesospim setups. mesoSPIM. Accessed on 19 june 2025. [Online]. Available: <https://mesospim.org/setups/> [Cited on page 13.]
- [29] J. R. C. Keller Lab. Isoview light-sheet microscope. Howard Hughes Medical Institute. Accessed on 19 june 2025. [Online]. Available: <https://www.janelia.org/archive/isoview-light-sheet-microscope-0> [Cited on page 13.]
- [30] O. Initiative. (2025) Gallery – openspim. Accessed on 19 june 2025. [Online]. Available: <https://openspim.org/Gallery> [Cited on page 13.]
- [31] mesoSPIM Initiative. (2025) The mesospim gallery. Accessed on 19 june 2025. [Online]. Available: <https://mesospim.org/gallery> [Cited on page 13.]
- [32] O. community. Who has an openspim. OpenSPIM. Accessed on 19 june 2025. [Online]. Available: <https://openspim.org/index> [Cited on page 12.]
- [33] mesoSPIM Initiative. The mesospim initiative. mesoSPIM. Accessed on 19 june 2025. [Online]. Available: <https://mesospim.org> [Cited on page 12.]
- [34] mesoSPIM Initiative. (2025) About mesospim. mesoSPIM. Accessed on 19 June 2025. [Online]. Available: <https://mesospim.org/about/> [Cited on page 12.]
- [35] O. Imaging. (2025) Micro-manager open source microscopy software. Open Imaging. Accessed on 19 june 2025. [Online]. Available: <https://micro-manager.org/> [Cited on pages 13 and 31.]
- [36] O. community. Welcome to μopenspim. OpenSPIM. Accessed on 19 june 2025. [Online]. Available: <https://openspim.org/micro-openspim> [Cited on page 13.]
- [37] M.-C. Developers, “Microscope-cockpit,” 2024, accessed on 2 july 2025. [Online]. Available: <https://microscope-cockpit.org/> [Cited on pages 14 and 31.]
- [38] C. Corp. Obis lx/lx laser box. . Accessed on 20 june 2025. [Online]. Available: https://coherentinc.my.site.com/Coherent/lasers/laser-accessories/obis-accessories/1228877?cclcl=en_US [Cited on pages 15 and 16.]
- [39] Coherent Corp. (2025) Obis lx 405 nm 100 mw laser. Coherent, Inc. Accessed on 20 June 2025. [Online]. Available: https://coherentinc.my.site.com/Coherent/1284371?cclcl=en_US [Cited on page 16.]

- [40] Coherent, Corp. (2023) Obis Is 488 nm 100 mw laser. Coherent, Inc. Accessed on 20 June 2025. [Online]. Available: https://coherentinc.my.site.com/Coherent/1226419?cclcl=en_US [Cited on page 16.]
- [41] Coherent Corp. (2025) Obis Is 561 nm 100 mw laser. Coherent, Inc. Accessed on 20 June 2025. [Online]. Available: https://coherentinc.my.site.com/Coherent/1253301?cclcl=en_US [Cited on page 16.]
- [42] Coherent, Corp. (2025) Obis Is 640 nm 100 mw laser. Coherent, Inc. Accessed on 20 June 2025. [Online]. Available: https://coherentinc.my.site.com/Coherent/1185055?cclcl=en_US [Cited on pages 15 and 16.]
- [43] I. Coherent, “Obis lx/lx datasheet,” , , Tech. Rep. Spec. 34232, May , revision 1 (May 2017). [Online]. Available: <https://www.coherent.com/resources/datasheet/lasers/obis-family-ds.pdf> [Cited on page 16.]
- [44] SCANLAB GmbH. (2025) SCANcube 7. Accessed on 20 june 2025. [Online]. Available: <https://www.scanlab.de/en/products/scan-systems/scancube/standard-series/scancube-7> [Cited on page 17.]
- [45] S. GmbH, *RTC 5 PC Interface Board Installation and Operation Manual, Rev. 1.9 e*, <https://www.manualslib.com/manual/2909604/Scanlab-Rtc-5-Pc-Interface-Board.html>, SCANLAB GmbH, 2020, accessed on 2 July 2025. [Cited on pages 17 and 27.]
- [46] ScanLab, “Rtc5 control board product brochure,” Product brochure, june 2025, available at <https://www.scanlab.de/en/products/control-electronics/rtc-control-boards>. [Cited on page 17.]
- [47] P. I. P. G. . C. KG, *M-110.xxx1 / M-111.xxx1 / M-112.xxx1 Microtranslation Stage: User Manual*, , September 2018, Document ID: M110T0032-EN. [Cited on pages 17 and 18.]
- [48] P. P. Instrumente, *DT-34 Precision Rotation Stage: User Manual*, , . [Cited on pages 17 and 18.]
- [49] P. P. Instrumente). M-110, m-111, m-112 compact linear stage. . Accessed on 20 june 2025. [Online]. Available: <https://www.physikinstrumente.com/en/products/linear-stages/stages-with-stepper-dc-brushless-dc-bldc-motors/m-110-m-111-m-112-compact-linear-stage-412418433> [Cited on page 18.]

- [50] P. I. P. S. . C. KG. (2025) C-884.4dc, c-884.6dc motion controller for dc motors, 4 or 6 axes. Accessed on 20 june 2025. [Online]. Available: <https://www.physikinstrumente.com/en/products/controllers-and-drivers/motion-controllers-drivers-for-linear-torque-stepper-dc-servo-motors/c-8844dc-c-8846dc-motion-controllers-for-dc-motors-900707> [Cited on page 18.]
- [51] Z. T. Inc. X-fwr-e series. . Accessed on 20 june 2025. [Online]. Available: <https://www.zaber.com/products/optical-mounts/X-FWR-E> [Cited on pages 18 and 19.]
- [52] H. P. K.K., *ORCA-Flash4.0 V3 Digital CMOS Camera C13440-20CU for Life Science*, , , 2025, cat. No. SCAS0120E02, February 2017. [Online]. Available: <https://camera.hamamatsu.com/eu/en/product/camera/C13440-20CU.html> [Cited on pages 19 and 26.]
- [53] Lifesciencemarket. Hamamatsu - orca-flash4.0 v3 digital cmos camera: C13440-20cu. Online product page. Accessed on 20 june 2025. [Online]. Available: <https://lifesciencemarket.com/en/hamamatsu-orca-flash4-0-v3-digital-cmos-camera-c13440-20cu.html> [Cited on page 19.]
- [54] Picodent, “Eco-sil,” , accessed on 29 june 2025. [Online]. Available: https://www.picodent.de/artikel/1315-eco_sil.html?akt=in&ab=2&id=1315&ang=&ARTIKEL=eco-sil&scrollTop=0&farbe_a=wei%C3%9F%2Ffila&vpeinheit_a=2+x+1+kg+Flasche&anz=0 [Cited on page 25.]
- [55] T. E. D. . S. Corporation, “Tbd62781apg, tbd62781afwg: 8-ch source type dmos transistor array datasheet,” Toshiba Semiconductor & Storage, Tokyo, Japan, Datasheet TBD62781APG / TBD62781AFWG, March 2017, p-DIP18 / P-SOP18 package; published 2017-03-29. [Online]. Available: https://toshiba.semicon-storage.com/info/TBD62781APG_datasheet_en_20170329.pdf?did=58397&prodName=TBD62781APG [Cited on page 28.]
- [56] B. A. Hall *et al.*, “Python-microscope: high performance microscope control,” *Journal of Cell Science*, vol. 134, no. 19, 2021, accessed on 2 July 2025. [Cited on pages 32 and 36.]
- [57] P. S. Foundation, *Python 3.12 Documentation*, Python Software Foundation, World Wide Web, 2023, accessed on 2 july 2025, <https://docs.python.org/3.12/>. [Cited on page 32.]

- [58] H. G. Arsenault, “Why c++ is not very fit for gui programming,” 2000, accessed on 2 July 2025. [Online]. Available: <https://arxiv.org/abs/cs/0001003> [Cited on page 32.]
- [59] M. Docs, “Wpf and win32 interoperation,” <https://learn.microsoft.com/en-us/dotnet/desktop/wpf/advanced/wpf-and-win32-interoperation>, 2025, accessed on 2 July 2025. [Cited on page 32.]
- [60] T. L. Nicholas Sofroniew *et al.*, *napari Documentation*, napari Contributors, 2022, accessed on 2 july 2025, <https://napari.org/>. [Cited on page 32.]
- [61] O. M. Environment, *OME-Zarr Documentation (version 0.4)*, Open Microscopy Environment, World Wide Web, 2023, accessed 2025-07-02, <https://ome-zarr.readthedocs.io/en/stable/>. [Cited on pages 33 and 53.]
- [62] O. M. E. (OME), *OME-NGFF: Next-Generation File Formats for Bioimaging, Specification Version 0.4*, <https://ngff.openmicroscopy.org/0.4/index.html>, 2022, accessed on 2 july 2025.
- [63] Z. Developers, “Zarr-python documentation, version 3.0.8,” <https://zarr.readthedocs.io/en/stable/>, 2025, accessed on 2 July 2025. [Cited on page 33.]
- [64] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, and A. Cardona, “Fiji: an open-source platform for biological-image analysis,” *Nature Methods*, vol. 9, no. 7, pp. 676–682, 2012. [Cited on page 33.]
- [65] Adobe Developers Association, *TIFF Revision 6.0 Specification*, Adobe Systems Incorporated Std., june 1992, final Version – TIFF 6.0. [Online]. Available: <https://www.itu.int/itudoc/itu-t/com16/tiff-fx/docs/tiff6.pdf> [Cited on page 33.]
- [66] Open Microscopy Environment, *OME-TIFF Specification, OME Model 6.3.1*, Open Microscopy Environment, Glencoe, IL, USA, 2024, online technical specification for OME-TIFF, embedding OME-XML metadata in TIFF/BigTIFF images. [Online]. Available: <https://docs.openmicroscopy.org/ome-model/6.3.1/ome-tiff/specification.html> [Cited on page 33.]
- [67] P. Developers, “Pyvisa: Control your instruments with python,” <https://pyvisa.readthedocs.io/>, 2024, accessed on 2 July 2025. [Cited on page 37.]

- [68] P. I. P. G. . C. KG, “Pipython: Python interface to physik instrumente motion controllers,” <https://pypi.org/project/pipython/>, 2024, accessed on 2 July 2025. [Cited on page 40.]
- [69] K. Lukin, “pylablib: Laboratory device control in python,” <https://pylablib.readthedocs.io/>, 2023, accessed on 2 July 2025. [Cited on page 42.]
- [70] C. R. Harris, K. J. Millman, S. J. van der Walt *et al.*, “Array programming with numpy,” *Nature*, vol. 585, pp. 357–362, 2020, accessed on 2 July 2025. [Cited on page 53.]
- [71] M. Fletcher and Contributors, “Pyopengl: Opengl bindings for python,” <http://pyopengl.sourceforge.net/>, 2024, accessed on 2 July 2025.
- [72] O. Team, *OpenCV: Open Source Computer Vision Library*, <https://opencv.org/>, 2024, accessed on 2 July 2025.
- [73] T. Lambert and contributors, “superqt: Supercharged qt widgets,” <https://github.com/pyapp-kit/superqt>, 2024, accessed on 2 July 2025.