

Development, Characterisation and Debugging of a custom Light Sheet Fluorescence Microscope

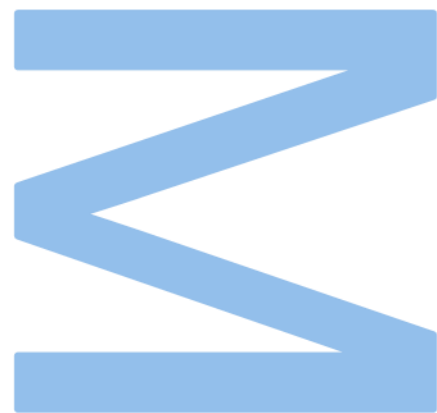
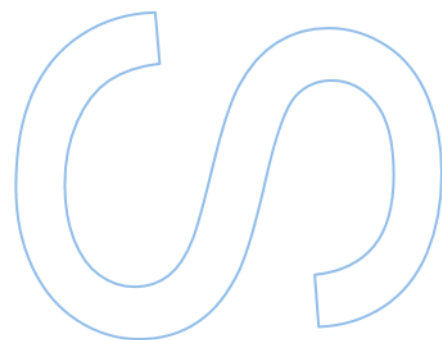
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Dedicated to my family

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Resumo

Esta dissertação apresenta o desenvolvimento, a caracterização e o debugging de um microscópio de fluorescência por folha de luz (*Light Sheet Fluorescence Microscope* – LSFM). Este trabalho foi desenvolvido na plataforma científica *Advanced Light Microscopy* (ALM) do i3S – Instituto de Investigação e Inovação em Saúde. O principal objetivo consistiu em expandir o software de controlo existente, melhorando a aquisição de imagem, a avaliação do desempenho óptico e a rastreabilidade operacional do sistema.

Uma das principais contribuições consistiu na implementação de um modo de aquisição confocal baseado na sincronização entre o varrimento do feixe de excitação e o modo de leitura por rolling shutter da câmara. Esta abordagem permite restringir a deteção pela câmara à região iluminada pela folha de luz, reduzindo a contribuição de fluorescência fora de foco e melhorando o contraste em comparação com o modo widefield baseado em global shutter. A comparação experimental entre os dois modos, realizada em diferentes amostras biológicas, demonstrou uma redução de luz proveniente dos planos fora de foco e uma melhor definição das diferentes de estruturas no modo confocal.

Foi também desenvolvido um modo de controlo de qualidade integrado no software. Este permite caracterizar a folha de luz através do ajuste gaussiano de perfis de intensidade, estimando parâmetros como a inclinação do feixe, a posição de foco e a largura a meia altura (FWHM) no foco. O sistema permite ainda gerar relatórios em PDF, ficheiros de dados estruturados e imagens anotadas, fornecendo uma ferramenta prática para alinhamento, monitorização e validação rotineira do microscópio. Complementarmente, foram realizados estudos de uniformidade de campo e de registo cromático usando soluções fluorescentes homogéneas e beads fluorescentes multicolores, tendo sido identificadas limitações associadas ao alinhamento óptico e à coincidência espacial entre canais.

Por fim, foi implementado um sistema de registo assíncrono de eventos para apoiar a depuração e a manutenção do software. Este regista eventos de hardware, eventos de software, erros de execução e exceções, permitindo reconstruir o estado do sistema durante cada sessão experimental. No seu conjunto, os desenvolvimentos apresentados contribuem para um ambiente de controlo mais robusto, reproduzível e orientado para o utilizador, ao mesmo tempo que evidenciam a importância do alinhamento óptico e da monitorização sistemática no desempenho de microscópios de folha de luz personalizados.

Palavras-chave: Microscopia Light Sheet, Microscopia Confocal, Rolling Shutter, Controlo de Qualidade, Log File, Debugging

Abstract

This dissertation presents the development, characterisation and debugging of a custom Light Sheet Fluorescence Microscope (LSFM). The work was carried out at the Advanced Light Microscopy (ALM) scientific platform of i3S – Institute for Research and Innovation in Health. The main objective was to expand the existing microscope-control software, improving image acquisition, optical performance assessment and operational traceability of the system.

One of the main contributions was the implementation of a confocal acquisition mode based on the synchronisation between the excitation-beam scan and the camera rolling-shutter readout mode. This approach restricts camera detection to the region illuminated by the light sheet, reducing out-of-focus fluorescence contributions and improving image contrast when compared with the widefield mode based on global shutter acquisition. Experimental comparison between the two modes, performed on different biological samples, showed reduced diffuse influence of out-of-focus and improved definition of local structures in confocal mode.

A quality-control mode was also developed and integrated into the microscope software. This mode enables light sheet characterisation through Gaussian fitting of intensity profiles, estimating parameters such as beam tilt, focus position and full width at half maximum (FWHM) at the focus. The system can also generate PDF reports, structured data files and annotated images, providing a practical tool for routine microscope alignment, monitoring and validation. In addition, field-uniformity and chromatic co-registration studies were performed using homogeneous fluorescent solutions and multicolour fluorescent beads, revealing limitations associated with optical alignment and spatial coincidence between channels.

Finally, an asynchronous event-logging system was implemented to support software debugging and maintenance. This system records hardware events, software events, runtime errors and exceptions, allowing the system state to be reconstructed during each experimental session. Overall, the developments presented in this dissertation contribute to a more robust, reproducible and user-oriented control environment, while also highlighting the importance of optical alignment and systematic monitoring in the performance of custom light sheet microscopes.

Keywords: Light sheet microscopy, confocal microscopy, rolling shutter, quality control, log file, debugging

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Symbols

E	Energy
h	Planck's Constant
c	Speed of light in vacuum
λ	Wavelength
f	Focal Length
w_0	Beam Waist
z_R	Rayleigh Length
b	Confocal Parameter

List of Abbreviations

AI	Artificial Intelligence
ALM	Advanced Light Microscopy
CNS	Central Nervous System
CW	Continuous-wave
DSLM	Digital Scanned Light Sheet
FOV	Field of View
FWHM	Full Width at Half Maximum
GUI	Graphical User Interface
LLSM	Lattice Light Sheet Microscopy
LS	Light Sheet
LSCM	Laser Scanning Confocal Microscopy
LSFM	Light Sheet Fluorescence Microscopy
NA	Numerical Aperture
PI	Physik Instrumente
PSF	Point Spread Function
QC	Quality Control
ROI	Region-of-Interest
SBR	Signal-to-Background Ratio
SDCM	Spinning Disk Confocal Microscopy
SNR	Signal to Noise Ratio
SPIM	Selective Plane Illumination Microscopy
WD	Working Distance

Preface

This dissertation was developed within the context of the Master in Engineering Physics at i3S – Institute for Research and Innovation in Health, specifically within the scientific platform of Advanced Light Microscopy (ALM) [1]. The platform provides advanced imaging services to researchers, supporting the acquisition of high-quality images of biological samples and assisting with subsequent image processing and analysis. It addresses a wide range of experimental requirements by offering technical expertise and access to state-of-the-art optical microscopy equipment.

The work was carried out with the objective of improving the microscope-control software and evaluating its impact on system operation, image acquisition, and optical performance. The project combines software development, optical characterisation, and experimental validation. In particular, it addresses the implementation of a confocal acquisition mode based on rolling-shutter synchronisation, the development of a dedicated quality-control workflow for light sheet characterisation, and the implementation of a logging system for debugging and operational traceability. These developments were motivated by the need to improve microscope usability, reproducibility, and robustness in a complex system composed of multiple hardware devices and software modules.

The dissertation is organised as follows. Chapter 1 introduces the relevant background on fluorescence microscopy, light sheet fluorescence microscopy, detection strategies, custom microscope-control software, and quality-control procedures. Chapter 2 describes the ALM microscope design, including its optical layout, hardware components, and software architecture. Chapter 3 presents the implementation of the rolling-shutter confocal acquisition mode and compares its performance with Widefield acquisition. Chapter 4 describes the quality-control tools developed for beam characterisation, field-uniformity analysis, and bead-based assessment. Chapter 5 presents the logging system implemented for microscope debugging. Finally, Chapter 6 summarises the main conclusions and outlines possible future improvements.

Overall, this work aims to contribute to a more reliable, traceable, and user-oriented control environment for the ALM light sheet microscope, while also providing tools to support routine system evaluation and future optimisation.

1. Background and State of the Art

1.1. Fluorescence in Microscopy

Since its introduction to biological research in the twentieth century, fluorescence has reshaped optical microscopy and has become a widely used technique in biotechnology. The ability to localise and quantify specific molecular species at the intracellular level renders fluorescence indispensable for modern imaging. Continued advances in fluorophore chemistry, alongside increasingly sophisticated strategies for imaging live and fixed specimens, have further consolidated the technique. A quantitative understanding of its photophysical basis is therefore essential to interpret performance limits in fluorescence imaging [2, 3].

1.1.1 Principles of Fluorescence

The phenomenon of fluorescence was first described by Sir George Gabriel Stokes in 1852, in his work "On The Change of Refrangibility of Light" [4, 5]. In modern terms, fluorescence is a form of photoluminescence, i.e. the emission of light from electronically excited states [3, 5].

Certain molecular species, hereafter referred to as *fluorophores* (see Subsection 1.1.2), absorbing light of certain wavelength and subsequently emit photons at longer wavelengths [6]. The emitted photons, therefore, have lower energy than the absorbed, and emission typically occurs within a few nanoseconds of excitation [7]. This difference between the absorption and emission wavelengths, as illustrated in Figure 1.1a, is known as the *Stokes shift* [3–5, 8]. This red-shift is consistent with Planck's relation (Eq. 1.1), which states that photon energy is inversely proportional to wavelength [9]. In practice, the Stokes shift primarily arises from rapid non-radiative relaxation (vibrational relaxation and internal conversion) to the lowest vibrational level of the excited state before photon emission [3, 5].

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

Fluorescence can also be interpreted using a Jablonski diagram, as shown in Figure 1.1b, which summarises radiative and non-radiative transitions between electronic states of a fluorophore [5].

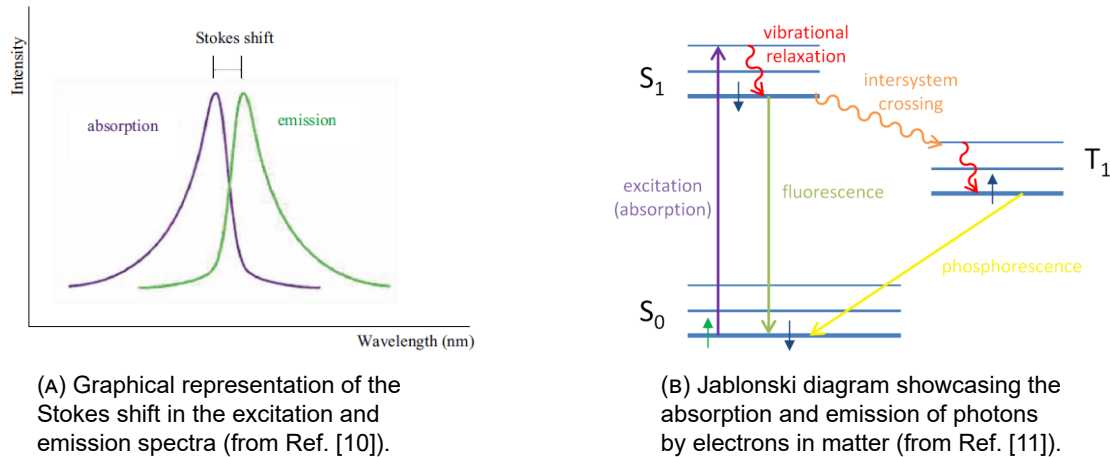


FIGURE 1.1: Stokes shift representation (left) and Jablonski diagram (right). Upon excitation, molecules transition from the ground singlet state (S_0) to an excited singlet state (S_1), followed by relaxation and fluorescence emission with photon energy corresponding to the S_1-S_0 gap (Eq. 1.1) [5, 11]. Non-radiative processes such as intersystem crossing to the triplet state (T_1) may also occur, leading to phosphorescence on longer timescales. Triplet-state population contributes to photobleaching and phototoxicity, while quenching processes reduce fluorescence intensity or lifetime.[3, 5]

1.1.2 Fluorophores and Fluorescent Probes

A fluorophore is a molecule that can absorb and emit photons, that is, a molecule that exhibits fluorescence [6]. Thousands of fluorescent probes have been developed to label or sense specific biological targets and micro-environments, enabling highly selective imaging in complex specimens [3]. In microscopy, fluorophores used for generating image contrast can be broadly categorised as intrinsic or extrinsic [5].

Intrinsic fluorophores are naturally occurring molecular species within the specimen that emit fluorescence, giving rise to sample autofluorescence [3]. Aromatic amino acids, NADH, flavins, derivatives of pyridoxyl and chlorophyll are common examples. In contrast, extrinsic fluorophores are added to the sample to provide fluorescence where none is present, or to change the spectral properties of the sample. Representative extrinsic fluorophores include quinine, fluorescein, and rhodamine [5].

To obtain high-quality images of a fluorophore in microscope, it is important to consider its excitation/absorption and emission spectra, which determine the compatibility between the chosen excitation line and the available optical filters [3, 7]. Figure 1.1a illustrates the Stokes shift between absorption and emission. In general, greater spectral separation facilitates rejection of the typically much brighter excitation light, whereas spectral overlap between fluorophores can lead to signal interference and inter-channel crosstalk in

multicolour imaging [3]. In this context, practical performance is often characterised using brightness and photostability metrics.

The fluorophore brightness (B_f) is a convenient proxy for signal intensity. Brightness is typically approximated by $\epsilon \times \Phi$ [12], where ϵ is the molar extinction (absorption) coefficient at the excitation wavelength, which relates the amount of light absorbed to the fluorophore concentration in solution, and Φ is the fluorescence quantum yield, defined as the ratio of the number of emitted photons to the number of absorbed photons. Photostability and susceptibility to photobleaching, the irreversible destruction of a fluorophore caused by prolonged or intense exposure to excitation light, impose practical limits on achievable acquisition duration and frame rate, particularly in time-lapse and volumetric imaging [3, 5].

In practice, fluorophore selection involves balancing signal generation against light-induced damage. Higher excitation irradiance can improve signal, but accelerates photobleaching and may exacerbate phototoxicity in live specimens [3, 7]. Some fluorophores, such as synthetic dyes (e.g. fluorescein, rhodamine and Alexa Fluor dyes), exhibit high brightness and can tolerate relatively high excitation intensities. In contrast, fluorescent proteins offer greater experimental flexibility, as they can be genetically encoded to enable selective targeting of specific regions or structures within the specimen. However fluorescent proteins have their own set of problems like how they react to light and how they overlap with other light [3]. Finally, red-shifted fluorophores can reduce background from autofluorescence and scattering in thicker samples, provided that filters and detector response remain compatible with the selected spectral window [3, 7].

1.1.3 Fluorescence in Biological Research

In biological research, fluorescence has become increasingly important owing to its ability to provide specific contrast in samples through selective labelling of structures. Fluorescence microscopy can achieve high levels of sensitivity and supports multicolour imaging by combining fluorophores with distinct spectral signatures [3, 7].

Immunofluorescence is one of the most widely used biological techniques for fluorescence microscopy. To visualise a cell or tissue of interest, the specimen is labelled with an antibody conjugated to a fluorophore, either directly or through the use of a secondary antibody. In direct labelling, the primary antibody is conjugated directly to the fluorophore, while in indirect labelling, an unconjugated primary antibody is first applied, followed by

a fluorophore-conjugated secondary antibody that recognises and binds to it (see Appendix A).

Beyond immunofluorescence in fixed samples, fluorescence is also used in live specimens through fluorescent proteins and synthetic probes. Fluorescent proteins enable direct fusion to proteins of interest, providing cell-type and subcellular targeting, whereas synthetic probes can be engineered either for localisation or for sensing micro-environmental parameters such as pH or ion concentrations [3, 7].

The choice between live and fixed-sample imaging reflects a trade-off between physiological relevance and experimental controllability. Fixed specimens provide structural stability, reduce motion artefacts, and allow extensive sample preparation (including long acquisitions and, when necessary, optical clearing), but they necessarily sacrifice dynamic information. Live imaging enables the observation of biological processes in real time; however, it introduces additional constraints related to photobleaching, phototoxicity, and the need to maintain physiological conditions throughout the acquisition [3, 7]. Consequently, experimental planning often hinges on managing the available photon budget: increasing the excitation dose can improve signal, but it typically accelerates bleaching and may compromise specimen viability, especially when speaking about live imaging [3, 7].

Biological specimens also impose optical limitations that affect image quality and quantitative interpretation. Thick samples are challenging for the majority of the microscopy techniques. These samples generate substantial out-of-focus light, and absorb, and scatter light, which can lead to the redistribution of emitted photons such that fluorescence appears displaced from its true origin, thereby degrading contrast. Endogenous molecular autofluorescence can further elevate background noise and contribute to spectral interference, particularly in multicolour experiments, emphasising the need for careful spectral selection and background control [3, 6]. This motivates the use of optical-sectioning approaches that reject, or do not lead to the formation of out-of-focus light, resulting in images with improved contrast [3]. Optical sectioning can be achieved using confocal microscopy, multiphoton microscopy, and light sheet microscopy [13].

Confocal microscopy achieves optical sectioning by using a pinhole aperture to reject out-of-focus light, enabling the acquisition of thin optical sections and the reconstruction

of three-dimensional structures with improved contrast and resolution compared to wide-field fluorescence microscopy. Two main implementations are widely used: Laser Scanning Confocal Microscopy (LSCM) and Spinning Disk Confocal Microscopy (SDCM). In LSCM, a focused laser beam scans the sample point by point (which makes the process of image acquisition slow) with emitted fluorescence detected through the pinhole, offering high spatial resolution, flexible spectral configuration, and compatibility with a wide range of fluorophores. SDCM uses a rotating disk containing multiple pinholes arranged in a spiral pattern, allowing simultaneous illumination and detection of many points across the sample, which greatly increases imaging speed and reduces photobleaching and phototoxicity. However, this parallelised acquisition comes at the cost of a moderate reduction in spatial resolution and optical sectioning efficiency, as well as reduced performance in thick or highly scattering samples due to cross-talk between pinholes [14]. Therefore, while LSCM is better suited for high-resolution imaging of fixed samples or slower biological processes, SDCM is the preferred choice for live-cell imaging and the capture of rapid dynamic events where speed and sample preservation are the primary concerns.

In multiphoton microscopy, near-infrared wavelengths provide deeper tissue penetration and reduced scattering, making it the method of choice for imaging thick tissues and *in vivo* preparations. The simultaneous absorption of two or more photons confines excitation intrinsically to the focal plane, and so there is no out-of-focus formation, photobleaching, or phototoxicity [15]. However, the technique requires costly pulsed infrared laser sources and offers lower imaging speed, as it depends on scanning the sample point by point.

1.2. Light Sheet Fluorescence Microscopy (LSFM)

Light Sheet Fluorescence Microscopy (LSFM) is a general name for a constantly growing family of planar illumination techniques. It traces its origins to ultramicroscopy in the early twentieth century, where planar side illumination was used to visualise individual particles [16]. The first widely cited implementation combining plane illumination with fluorescence for three-dimensional imaging of macroscopic biological specimens was introduced in 1993, when Voie, Burns and Spelman applied orthogonal plane fluorescence optical sectioning of an intact excised guinea-pig cochlea [17, 18]. In 2004, Huisken *et al.* introduced Selective Plane Illumination Microscopy (SPIM), combining planar side illumination with orthogonal wide-field (camera-based) detection to enable fast optical sectioning for *in vivo* imaging of developing embryos [16, 18, 19].

1.2.1 Principles of LSFM

LSFM consists of illuminating the sample with a thin sheet of light, usually referred to as a Light Sheet (LS), but only in the portion of the sample being imaged [20]. The LS is formed by laser light either shaped into a hyperbolic "sheet" of illumination, or approximated by scanning a weakly focused low Numerical Aperture (NA) beam in the lateral directions. A key feature of this technique is that illumination and detection are performed along different optical axes, which are typically arranged orthogonally (Figure 1.2) to maximise detection efficiency while minimising out-of-focus fluorescence [16].

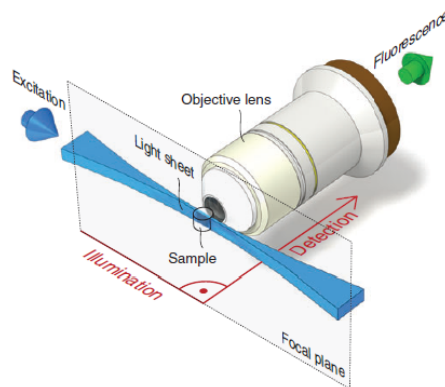


FIGURE 1.2: Schematic illustration of LSFM. The illumination beam is shown in blue, and the detection axis is indicated by the green arrow (from Ref. [21]).

The main benefits of LSFM arise from the fact that excitation is largely confined to the focal region, thereby avoiding unnecessary out-of-focus excitation that would otherwise generate background fluorescence and degrade the quality of the in-focus signal. Consequently, LSFM can typically operate with lower excitation irradiance while using planar illumination to improve the Signal to Noise Ratio (SNR) with minimal exposure of the specimen. Scanning the LS or translating the specimen along the axial direction then enables rapid and minimally invasive three-dimensional imaging, resulting in reduced photobleaching and phototoxicity [16, 21, 22].

Despite these advantages, LSFM can be difficult to implement, mainly because of the difficulty of sample mounting and preparation, and the need for two or more objectives, which hinders the beam alignment process [16]. In scattering or absorbing samples, shading may occur, and the achievable sectioning is limited by the balance between the thickness of the light sheet and the usable Field of View (FOV) [21, 22]. These limitations can be overcome with some illumination and detection strategies such as those in section 1.3.

1.2.2 Light-Sheet Beam Formation and Characterization

The most important aspect of the LSFM technique is the light sheet (LS) itself. The LS can be formed from a static planar sheet, as in the original SPIM technique, or from the scanning beam over time. It is important to know that creating a perfect planar LS is impossible, it can only be approximated over a given range [16].

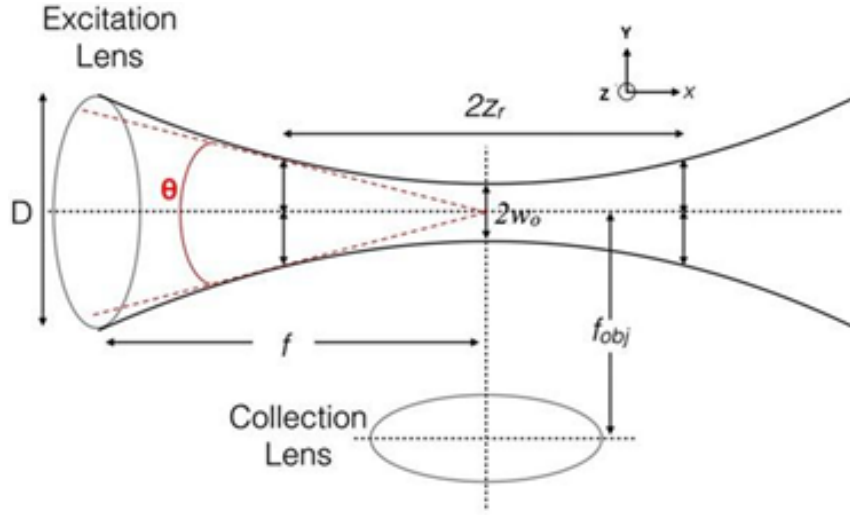


FIGURE 1.3: Illustration of the Gaussian beam (Adapted from Ref. [20]).

When the beam propagates along the x -axis, a Gaussian profile is formed in the yz -plane. As shown in Figure 1.3, the beam waist w varies along the propagation direction and follows Eq. 1.2, where $w(x)$ denotes the $1/e^2$ beam radius of the Gaussian intensity profile in the transverse plane [23].

$$w(x) = w_0 \sqrt{1 + \left(\frac{x}{z_R}\right)^2} \quad (1.2)$$

In Eq. 1.2, w_0 denotes the beam waist at focus, corresponding to the radius of the most tightly confined region of the LS. This quantity can be estimated using Eqs. 1.3 and 1.4 for large x [16, 24], where f is the focal length of the illumination optics, λ is the wavelength in medium, and D_{lens} is the effective aperture diameter (diaphragm) of the illumination optics.

$$w_0 = \frac{1.4f\lambda}{4D_{\text{lens}}} \quad (1.3)$$

$$\theta \approx \frac{\lambda}{\pi w_0} \iff w_0 \approx \frac{\lambda}{\pi \theta} \quad (1.4)$$

In addition, Eq. 1.2 includes z_R , the Rayleigh length (see Eq. 1.5), which characterises the propagation distance over which the beam remains approximately collimated. It is commonly defined as the distance from the waist at which the beam radius increases by a factor of $\sqrt{2}$ [23, 25].

$$z_R = \frac{\pi w_0^2}{\lambda} \quad (1.5)$$

Of particular interest is the confocal parameter b of a LS, which specifies the distance along the propagation direction over which the sheet can be regarded as providing an approximately homogeneous planar illumination. Within this range, the sheet thickness is minimal at the beam waist and increases towards either end of the confocal parameter [16]. The confocal parameter therefore sets the maximum usable extent of the LS along the propagation direction and is defined by Eq. 1.6.

$$b = 2z_R = \frac{2\pi w_0^2}{\lambda} \quad (1.6)$$

This equation refers to the propagation of light in a medium with refractive index n . The confocal parameter is also given by Eq. 1.7, where λ_0 is the wavelength in vacuum/air ($\lambda = \frac{\lambda_0}{n}$).

$$b_n = \frac{2\pi w_0^2}{\lambda_0} \cdot n \quad (1.7)$$

In practice, the LS thickness is not usually reported as $2w_0$, as illustrated in Figure 1.2. Instead, the thickness is typically quantified using the Full Width at Half Maximum (FWHM) of the beam intensity profile at the focus (see Eq. 1.8) [26].

$$t_{\text{FWHM}} = \sqrt{2 \ln 2} \cdot w_0 \quad (1.8)$$

In addition, it is important to know the image FOV, which can be approximated by Eq. 1.9 [20]. Thus, decreasing w_0 improves axial sectioning but reduces z_R and therefore shortens the usable FOV.

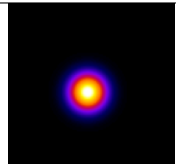
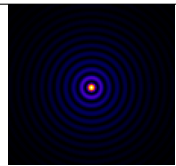
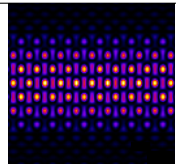
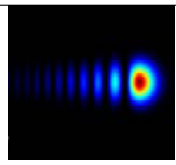
$$FOV = 2z_R \quad (1.9)$$

It is also important to have an approximate relation for the axial resolution of the final image. This relation is given by Eq. 1.10 [20], where D_{illum} is the diameter of the illumination objective and NA is the numerical aperture of the illumination optics. This expression indicates that increasing the illumination NA leads to a smaller beam waist (w_0) and a shorter Rayleigh length (z_R). Consequently, a lower illumination NA yields a thicker light sheet but increases its usable axial extent (i.e. a longer propagation-invariant region) of the LS.

$$R_{\text{axial}} = 2w_0 = \frac{4\lambda f}{\pi D_{\text{illum}}} = 2 \frac{n\lambda}{\pi \text{NA}} \quad (1.10)$$

Depending on the LSFM architecture employed (Subsection 1.2.3) and the illumination strategy adopted (Subsection 1.3.1), different beam types may be used or engineered to illuminate the specimen, including Gaussian, Bessel, lattice, and Airy beams. The choice of beam type –and, consequently, the illumination strategy, –is motivated by the specific beam characteristics relevant to each application, as summarised in Table 1.1.

TABLE 1.1: Qualitative comparison of light sheet beam types.[16, 27–31]

Beam Type	Usable FOV	Sheet Thickness	Background excitation	Beam Format
Gaussian	Diffraction-limited; thin sheet $\Rightarrow$ smaller FOV.	Minimum at waist; thickens with propagation.	Low out-of-plane excitation; FOV limited for very thin sheets.	
Bessel	Extended range (quasi-invariant core).	Very thin central lobe.	High background due to side lobes (photodamage risk).	
Lattice	Extended effective FOV	Ultrathin; improved axial sectioning.	Lower background than Bessel (side-lobe suppression).	
Airy	Extended FOV vs Gaussian; supports high-throughput.	Asymmetric profile; effective confinement over a longer range.	Moderate; tails can add background unless handled.	

1.2.3 LSFM Architectures

As mentioned at the beginning of Section 1.2, LSFM is a family of microscopy architectures based on the general principle of excitation/illumination of the sample along a distinct axis, usually orthogonal to detection. Over the development of LSFM techniques, multiple architectures have emerged to improve image quality by modifying the arrangement of objectives, the strategy used to generate the LS, the acquisition scheme, and the robustness to scattering and shadowing artefacts. Therefore, there are different architectures such as Selective Plane Illumination Microscopy (SPIM), mSPIM (multi-view SPIM), Digital Scanned Light Sheet (DSLM), and Lattice Light Sheet Microscopy (LLSM).

In the **SPIM** architecture, the LS is generated statically, typically from a cylindrical lens, and the fluorescence is collected orthogonally by camera detection. This parallel camera acquisition favours high volumetric speed. This architecture is susceptible to shadows/stripes in dispersive media due to the reduced angular diversity of the illumination. The uniformity of the illumination tends to depend on the sample. Beam alignment tends to be robust between the arms, and the mounting can be restrictive, requiring lateral access and specific mounts (like capillaries, agarose, specific supports) [20, 22].

In **mSPIM**, the LS is pivoted within the illumination plane, providing multi-directional illumination. This can improve illumination uniformity and reduce shadowing artefacts through temporal averaging. However, the rotation within the illumination plane must be fast relative to the camera integration (exposure) time, which increases system complexity and makes calibration more demanding. Sample mounting constraints are broadly similar to those of SPIM [32].

DSLM differs from SPIM primarily in that the LS is generated by scanning a focused beam, which can improve illumination uniformity and mitigate shadowing, particularly when combined with a rolling-shutter readout (see Subsection 1.3.2). The achievable volumetric imaging speed depends on the scan rate, the exposure time, and the camera readout. Sample mounting is similar to the preceding architectures, but optical alignment is more complex, mainly due to the need for precise synchronisation between the scanner, the laser, and the camera [20, 22, 32].

LLSM employs lattice illumination to achieve high-quality optical sectioning and strong spatiotemporal performance, making it well-suited to high-speed volumetric live imaging with good illumination uniformity. However, LLSM is more difficult to calibrate and is

more sensitive to misalignment. Sample mounting is generally most favourable for small volumes and single-cell preparations. Applications of LLSM include the study of phagocytosis and other rapid three-dimensional dynamics [22, 33].

1.3. Illumination and Detection Strategies in Light-Sheet Microscopy

1.3.1 Illumination Strategies

The LLSM architectures available (Subsection 1.2.3) strongly influence the final image quality and are therefore closely linked to the choice of illumination strategy. Selecting an appropriate illumination approach requires considering how well it controls excitation uniformity, its robustness to shadowing, its penetration depth in the specimen, the photodamage associated with out-of-plane excitation, and its instrumental complexity. The most common illumination strategies are closely associated with the underlying beam type (see Table 1.1), including Gaussian beams (static and scanned), dual-sided illumination, Bessel beams, and lattice illumination.

The simplest way to generate a LS is to shape a Gaussian beam into a quasi-planar illumination using a cylindrical lens, an approach that is common in SPIM. Once aligned, this configuration tends to be mechanically and optically stable, and is therefore comparatively less complex. However, it is important to note that a thinner LS improves axial sectioning but reduces the usable FOV [20]. Moreover, because the sheet is static, the angular diversity of illumination is limited, making the system more susceptible to shadowing artefacts, which can degrade illumination uniformity and image contrast [20, 22].

In addition to using a static Gaussian beam, the same beam type can also be implemented in a scanned configuration, as mentioned for DSLM in Subsection 1.2.3. Scanning promotes temporal averaging of the illumination, improving uniformity and reducing shadowing artefacts, particularly when combined with appropriate camera readout strategies (see Subsection 1.3.2). This approach, however, increases system complexity because it requires stable synchronisation between the scan and detection process [20, 32].

In these cases, illumination can be implemented in a dual-sided configuration. This strategy reduces shadowing by illuminating the specimen from two opposing directions, which tends to improve uniformity and to mitigate dark regions associated with attenuation-induced shadows. The main trade-off is increased system complexity, since alignment becomes more demanding and power balancing becomes important to avoid an unnecessary increase in the total excitation dose [20].

Beyond strategies based on Gaussian beams, Bessel can also be employed. As indicated in Table 1.1, a Bessel beam exhibits a central lobe that remains approximately propagation-invariant over longer distances, with an effective waist comparable to that of a Gaussian beam. Bessel beams are also attractive because they exhibit partial self-reconstruction, which increases robustness to shadowing and can improve penetration in scattering specimens. However, the presence of significant side lobes can excite fluorophores outside the focal plane, thereby increasing background signal and raising the risk of photodamage [20].

As already mentioned in subsection 1.2.3 and in Table 1.1, lattice illumination can be used. This type of illumination achieves higher optical section quality and spatio-temporal performance, reducing out-of-plane excitation. In practice, it is effective for fast volumetric live imaging. However, this strategy is more complex in terms of calibration and alignment stability, making it more demanding to implement [20, 29].

Alongside selecting an appropriate illumination strategy, combining it with a robust detection (see Subsection 1.3.2) ensures that a high-quality image of the sample is obtained for analysis.

1.3.2 Detection Strategies

As mentioned in subsection 1.3.1, choosing the best detection strategy in LSFM is just as important as choosing the illumination. As is known, the final quality of an image depends on the photons that contribute to its formation on the sensor, especially when light is scattered, which can increase the background and reduce contrast. Signal-to-Background Ratio (SBR) is a critical parameter in experimental performance [20, 32].

One detection strategy is to use the camera in a *global shutter*. That is, all the camera's pixels start and end integration simultaneously. This approach is operationally simple and robust. With the camera in global shutter, fewer synchronization requirements are needed, direct exposure times are used, and there are fewer time lags within the FOV [34, 35]. However, all photons that hit the sensor during exposure contribute to the image (see Figure 1.4a), tending to collect more background, penalizing SBR and limiting SNR in background-dominated regimes [20, 32].

A powerful alternative is to operate the camera in *rolling-shutter* mode. In this mode, exposure and readout occur line by line (see Figure 1.4b). In a DSLM-type LSFM system, the LS is generated by scanning the beam; therefore, the beam position can be synchronised

with the sensor's active band. As a result, fluorescence originating from the illuminated region is accepted, whereas scattered and out-of-focus contributions are rejected, effectively acting as a "virtual pinhole" with a line geometry [32, 36]. It is important to note that the slit (or active-band) width directly determines the amount of detected background. Narrower slits provide stronger background rejection; however, they make the system more sensitive to misalignment and synchronisation errors. Conversely, wider slits admit more background, but they increase throughput and overall robustness by reducing sensitivity to small alignment deviations [35, 36].

The main practical consequence of using rolling shutter mode is the requirement to synchronize the beam scanning with the rolling shutter kinematics and with the illumination itself. This brings difficulties regarding the trigger and the calibration of the relative delay between signals, which can introduce artefacts into the image that are not correct [32, 36]. Furthermore, performance is limited by the sensor's read speed and time per line. In scenarios with fast dynamics or residual sample movement, rolling shutter can introduce temporal distortions within the frame that do not occur in global shutter mode [34, 35].

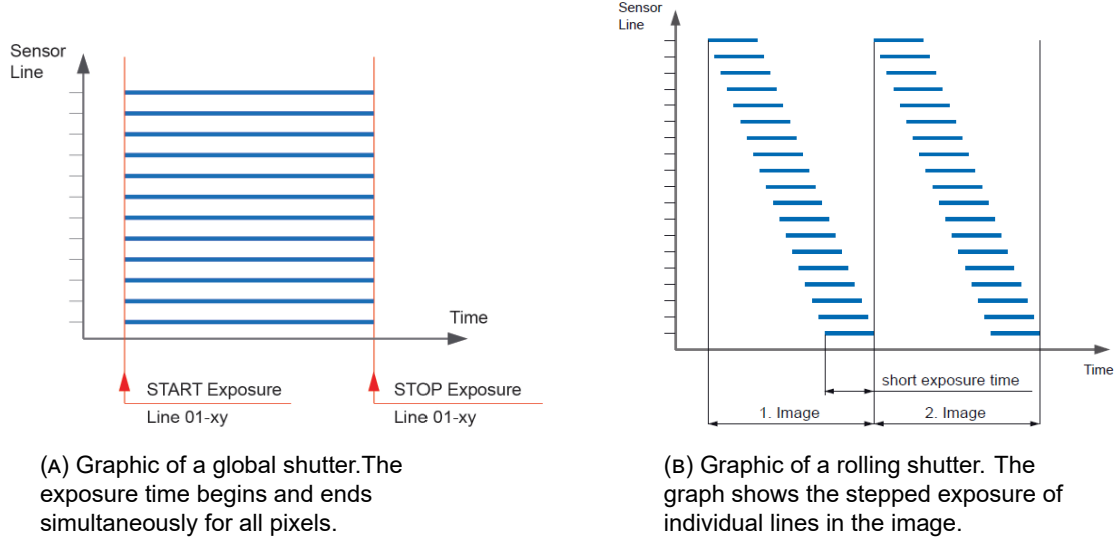


FIGURE 1.4: Graphics of global shutter(left) and rolling shutter(right) exposure in time (from Ref. [34]).

These detection capabilities place additional requirements on the control software and hardware (see Section 1.4).

1.4. Custom-Made Control Software for LSFM Systems

1.4.1 Existing Software

Over the years, and alongside the rapid growth of LSFM, new control methods have been developed in response to the increasing reliance on multiple hardware devices and to practical constraints related to inter-device synchronisation, real-time performance, data scalability, and sample-specific adaptations. This demand for specialised software has led to a diverse ecosystem of solutions, including commercial packages, open-source platforms, vendor-provided software, and fully customised frameworks tailored to specific experimental requirements.

Commercial platforms implement turnkey solutions that offer stable operation, high robustness, and complete integration between hardware and software. Representative examples include ZEISS ZEN [37], used in systems such as the ZEISS Lightsheet 7 [38] and the ZEISS Lightsheet Z.1 [39]; the Leica LAS X platform used with the STELLARIS DLS microscope [40]; and Nikon NIS-Elements [41]. These packages are commercial software suites for data acquisition and workflow management, enabling hardware control and multidimensional imaging. Despite their stability, such solutions often impose limitations on low-level extensibility and are typically not compatible with hardware outside the supported vendor ecosystem.

For these reasons, open-source tools provide more flexible and often more suitable alternatives for LSFM systems. *µManager* is a widely used platform for microscope hardware control and automation. Depending on the availability of device drivers for the instrumentation in use, Micro-Manager supports common acquisition modalities such as multichannel imaging, z-stacks, and time-lapse experiments [42]. Another open-source platform is *OpenSPIM*. It provides a modular framework for building configurable SPIM systems that can be shared with the community, thereby lowering the barrier to entry and enabling application-specific optimisation [43]. In the Python ecosystem, *python-microscope* enables control of microscopy devices, geared towards complex systems with timing requirements [44]. Finally, there is also *Microscope-Cockpit*, which features an extensible Python Graphical User Interface (GUI) for microscopes [45]. Despite this flexibility, scanning and multi-view approaches require strict synchronization and more specialized pipelines.

Manufacturers typically provide reliable access to device-specific functionalities, which are indispensable in both commercial and open-source control environments. Typical

examples are:

- *Hamamatsu DCAM-API* [46]
- *Basler Pylon SDK* [47]
- *Coherent OBIS Remote/Laser Box* [48]

The main limitation in these situations is that such systems often do not support the custom scanning and triggering sequences required by advanced LSFM implementations. In such cases, it becomes necessary to develop fully customised control software, which can be implemented in Python (e.g. using PyVISA, pyllablib, PySide6, and napari), LabVIEW, or C/C++. These bespoke architectures enable more precise synchronisation, tighter co-ordination across devices, and integrated acquisition–processing–visualisation pipelines with real-time feedback.

1.4.2 Requirements for Custom LSFM Control

LSFM integrates multiple hardware devices, including cameras, beam-scanning units (in DSLM implementations), motion stages, and lasers. Their operation must be coordinated, requiring precise timing as well as efficient data-flow management. Consequently, the control software must be designed to maximise system performance to obtain the highest-quality images.

One of the key requirements for an LSFM to operate reliably is precise synchronisation between acquisition and control events, such as laser on/off modulation, beam scanning, stage motion, and camera exposure/triggering. In practice, this entails orchestrating TTL/clock signals and well-defined relative delays in a reproducible manner, minimising jitter and ensuring temporal co-registration between illumination and detection [49].

Custom control must support real-time acquisition without frame loss. In practice, this requires a non-blocking multi-threaded architecture, buffers to control software RAM usage, and asynchronous writing to prevent continuous loops [49].

Furthermore, it is important that the data be interpretable and that there is operational logging that records laser power, detection and movement parameters, timestamps, and device states, as well as error events. So, the entire community working on microscopes can track these errors with standardized metadata models [49].

Finally, it is essential to monitor and document the LSFM system state. This includes recording the protocols used, the versions of the control software and integrated drivers, and the calibration procedures applied, because even small phase variations can degrade image quality and introduce systematic errors.

1.4.3 Data Types in Acquisition

Data generated in LSFM are typically multidimensional, comprising spatial dimensions (X , Y , Z) acquired through axial scanning. For dynamic acquisitions, it is common to additionally record time points (t), spectral channels (c), and tiled fields of view to cover larger sample areas. The *bit depth* (e.g. 8, 12/16 bits) and the effective acquisition dimensions (ROI, number of planes N_z , channels N_c , and time points N_t) directly determine the sustained data rate and the total data volume per experiment.

The way in which data are serialised and accompanied by metadata directly impacts portability, provenance preservation, and read/write performance. There are several file formats commonly used to store this type of bioimaging data. OME-TIFF [50], HDF5 [51], and the OME-NGFF/OME-Zarr ecosystem [52] represent different approaches to storing LSFM datasets in biological imaging.

However, the dimensions and formats chosen for storing the data can lead to storage problems, requiring compression strategies and naming and organization conventions on disk. Therefore, it is recommended that each acquisition be self-describing with deterministic names based on indexes in folders consistent with robust metadata storage, reducing copying problems and facilitating pipeline automation.

1.5. Quality Control

In microscopy, the quality and reproducibility of its operation are essential. This is achieved through Quality Control (QC) processes that monitor the instrument's performance over time, since subtle changes, such as misalignments and mechanical drifts, are natural and introduce systematic biases in measurements.

In LSFM, QC is particularly relevant owing to the strong dependence on the illumination–detection geometry. It is necessary to ensure proper alignment of the LS with the focal plane, a well-characterised Point Spread Function (PSF) with adequate axial and lateral resolution, and uniform illumination across the FOV. These factors directly influence image contrast and SNR, and consequently the overall fidelity of the acquired data. In addition,

stage drift and accurate spatial calibration (e.g. converting μm to pixels and vice-versa) are important for robust quantitative measurements [53, 54].

1.5.1 QC Tests

In LSFM, several tests are performed in QC processes, namely optics, detection, synchronization and stability tests.

In the **optics tests**, the LS thickness is verified through the FWHM (Eq. 1.8), and the variation of $w(x)$ along the propagation direction is quantified (Eq. 1.2). In addition, the usable extent of approximately homogeneous illumination is determined using z_R and b (Eqs. 1.5 and 1.6). Illumination uniformity is assessed within the expected FOV to detect misalignment or shading artefacts.

The **detection tests** focus on the imaging performance and detector characterisation. The PSF is measured using fluorescent beads (see Subsection 1.5.2) to obtain axial and lateral FWHM values. In parallel, the detector is characterised through offset maps, read noise measurements, and hot-pixel maps.

The **synchronization tests** evaluate the temporal coordination between system components. Relative delays between TTL signals are measured and subsequently validated through image-based tests. In addition, the rolling-shutter delay is adjusted to maximise image contrast.

In the **stability tests**, it is verified the long-term robustness of the system. These include confirming that the beams and the overall optical path remain properly aligned, as well as ensuring the absence of stage drift and verifying that the commanded positions keep the specimen within the FOV.

1.5.2 Sample to use in QC processes

During QC procedures, it is common to use different samples to evaluate distinct system functionalities. Sub-resolution fluorescent beads (250–500 nm [55]) are used to assess resolution by acquiring image stacks that allow estimation of the axial and lateral FWHM. Fluorescent slides are used to measure spatial variations across the FOV and to quantify illumination uniformity. Simple biological specimens can serve as end-to-end validation samples, for example, to assess shadowing artefacts under realistic imaging conditions.

1.5.3 Data Analysis in QC

During the QC process, it is common to acquire dedicated datasets, from which a QC report can be generated containing tables, plots, and logs summarising the required metrics. For LSFM systems, such reports should include resolution measurements (e.g. axial and lateral FWHM), illumination uniformity metrics over the measured FOV, and SNR estimates that can be used to fine-tune rolling-shutter synchronisation.

2. ALM Microscope Design

The custom LSFM system available at ALM is based on the DSLM architecture and comprises a single illumination and two detection paths. This chapter presents the complete architecture of the LSFM microscope, with particular emphasis on the hardware configuration, the optical layout, and the software framework developed to coordinate device control, image acquisition, and user interaction.

2.1. Microscope Devices

The LSFM implementation at ALM relies on the integration of multiple hardware subsystems responsible for beam activation and steering, mechanical sample positioning, filters selection, and image detection. In a DSLM configuration, these subsystems must operate in a coordinated manner, as the final image performance depends not only on the individual characteristics of each device but also on their temporal and functional compatibility.

2.1.1 Laser Box

The excitation system of the ALM LSFM is based on the Coherent OBIS LX/LS Laser Box (Figure 2.1e) [56]. The current implementation includes four lasers: OBIS LX 405 nm (Figure 2.1a) [57], OBIS LS 488 nm (Figure 2.1b) [58], OBIS LS 561 nm (Figure 2.1c) [59] and OBIS LX 640 nm (Figure 2.1d) [60].

The lasers are operated over a power range from 1 mW to 100 mW and are controlled via USB from the host computer. The OBIS platform supports both Continuous-wave (CW) operation and external modulation, with analog (0–5 V) and digital (up to 150 MHz) connections, although the available bandwidth depends on the specific model.



(A) OBIS LX 405 nm
(from Ref. [57]).



(B) OBIS LS 488 nm
(from Ref. [58]).



(c) OBIS LS 561 nm
(from Ref. [59]).



(d) OBIS LX 640 nm
(from Ref. [60]).



(E) OBIS LX/LS Laser
Box (from Ref. [56]).

FIGURE 2.1: Laser devices in use at the ALM light sheet microscope.

The laser output is delivered in free space and is subsequently redirected through a set of mirrors, including dichroics, towards the scanner. This multi-line excitation configuration provides spectral flexibility for fluorescence imaging and enables software-controlled integration with the remaining hardware subsystems.

2.1.2 Scanning Mirrors

Beam scanning is performed using a SCANcube 7 galvanometric scanner (Figure 2.2a) [61] driven by a ScanLab RTC5 control board (Figure 2.2b) [62], which is directly connected to the control computer's motherboard via PCI Express.



(A) SCANcube 7
galvanometric
scanner (from
Ref. [61]).



(B) ScanLab RTC5 Control
Board (from Ref. [62]).

FIGURE 2.2: Galvanometric scanning system installed at the light sheet microscope composed of a) SCANcube7 galvanometric scanner and a b) ScanLab RTC5 control board.

This scanner-based configuration is responsible for dynamically controlling the angular deflection of the excitation beam before reaching the illumination path, thereby enabling

the generation of an approximately planar LS used for fluorescence excitation. The Scan-Lab RTC5 board provides the control layer required to operate the galvanometric scanner and integrate it into the timing architecture of the microscope. In practice, the scanning parameters determine how the excitation beam is swept across the field of view and therefore influence the spatial extent and positioning of the generated LS.

2.1.3 Linear and Rotation Stages

Sample positioning in the ALM LSFM is achieved through a motorised stage subsystem comprising X , Y , and Z translation axes together with a θ rotation axis. The subsystem comprises the following Physik Instrumente (PI) stages:

- Two PI M-110.1DG1 stages provide motion in the XY plane with $5\text{ mm} \times 5\text{ mm}$ travel range (Figure 2.3a)[63];
- One PI M-112.1DG1 stage is used for axial Z motion with a 25 mm range (Figure 2.3b) [63];
- One PI DT-34 rotation stage allows controlled reorientation of the sample about the θ axis (Figure 2.3c) [64].

All stages are controlled with a PI C-884.4DC motor controller (Figure 2.3d) [65], which connects to the computer via USB. Stage control is integrated into the microscope software, which supports axis referencing routines to establish known reference positions, absolute positioning, fine and coarse step motion, and real-time position updates. Collision avoidance logic is also implemented to ensure safe operation of the motorised axes, with a 1 mm safety margin along the Z axis.



(A) PI M110.1DG1 mounted on top of another linear stage(from Ref. [63]).



(B) PI M-112.1DG1(from Ref. [63]).



(c) PI DT-34(from Ref. [64]).



(d) PI C-884.4DC(from Ref. [65]).

FIGURE 2.3: Linear (a,b) and Rotation (c) stages installed on the light sheet microscope, and the motion-controller (d).

This configuration enables controlled positioning of the sample with respect to the illumination and detection paths and is essential for the practical operation of the microscope.

2.1.4 Filter Wheels

Selection of spectral detection is performed using two motorised Zaber X-FWR-E filter wheels (Figure 2.4) [66], one associated with each detection path. Communication with the control computer is established via USB connection.



FIGURE 2.4: Zaber X-FWR-E filter wheel installed on the light sheet microscope (from Ref. [66]).

The filter wheel subsystem is responsible for selecting the emission filter, which determines the fluorescence wavelength band transmitted to the camera sensor, thereby rejecting unwanted spectral contributions. Each wheel accommodates ten 25 mm filters and supports switching times of 57 ms between adjacent positions, thereby impacting delays during filter changes in multichannel acquisitions. Filter selection is integrated into the microscope software and can be associated with the active excitation laser during acquisition.

The filters currently available in the ALM microscope and their spectral characteristics are listed in Appendix B.

2.1.5 Cameras

Image detection in the ALM microscope is performed using two Hamamatsu ORCA-Flash4.0 V3 C13440-20CU sCMOS cameras (Figure 2.5) [67–69], one associated with each detection path. This dual-camera configuration enables parallel image acquisition from the two detection arms and constitutes the final stage of the optical detection chain. Both cameras are connected to the control computer via a Camera Link interface, ensuring high-throughput data transmission.



FIGURE 2.5: Hamamatsu ORCA-Flash4.0 V3 C13440-20CU camera used at the light sheet microscope (adapted from Ref. [68, 69]).

Each camera provides a sensor format of 2048×2048 pixels with a pixel size of $6.5 \mu\text{m} \times 6.5 \mu\text{m}$ and an effective imaging area of $13.312 \text{ mm} \times 13.312 \text{ mm}$. This model also provides high quantum efficiency and full-resolution frame rates of up to 100 Hz, depending on the selected readout interface and acquisition settings [68, 69]. These characteristics make these cameras well-suited to LSFM applications, such as live-cell imaging and fast volumetric acquisition.

The camera output can be configured in 16-bit, 12-bit, or 8-bit modes, thereby allowing different intensity levels to be assigned to each pixel. A higher bit depth improves the dynamic range, enabling finer discrimination between intensity values. In addition, the camera supports reduced-resolution 2×2 and 4×4 in-camera binning modes, i.e. the combination of adjacent pixels through the summation of their values, which can improve the

SNR at the expense of spatial resolution, as well as through cropped Region-of-Interest (ROI) readouts [68, 69].

The ORCA-Flash4.0 V3 C13440-20CU supports internal and external triggering, making it compatible with synchronised control. In the microscope control software, two exposure modes are available: Global Shutter mode, in which the entire sensor is exposed simultaneously, and Light sheet mode, in which a rolling shutter is used and synchronisation with beam scanning can be implemented. In the latter case, out-of-focus light contributions can be reduced, thereby improving optical sectioning and image contrast. The operational implementation of the rolling-shutter mode and the corresponding widefield-style acquisition mode is discussed in Chapter 3.

2.2. Full System Architecture

The hardware devices described in Section 2.1 form an integrated optical and controlled system that enables fluorescence excitation, beam scanning, and image detection in a coordinated manner.

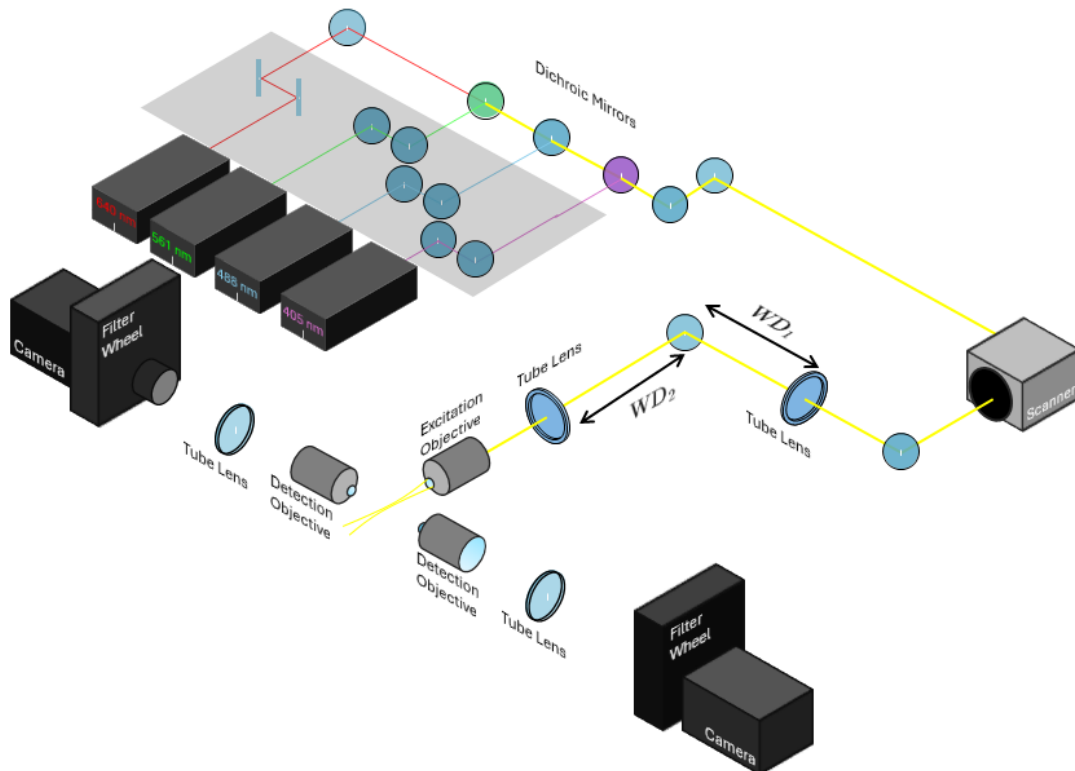


FIGURE 2.6: Schematic illustration of the light sheet microscope architecture showing the three main subsystems. The diagram presents the complete beam path up to the sample (the chamber is not represented for clarity, although the sample would be located at the position of the depicted light sheet (LS)) and the fluorescence emission path to the cameras (adapted from Ref. [70]).

In the ALM microscope, image formation results from the interaction between its different subsystems. Conceptually, the system can be divided into three interdependent subsystems: the reflection optics system, located immediately after the laser outputs; the illumination path system; and the dual detection paths, which include the sample region where image acquisition takes place.

A complete schematic representation of the system is illustrated in Figure 2.6, while real images of the complete system are provided in Appendix C.

2.2.1 Reflection Optics

The beam exits the laser box in free space and is directed through a set of steering and dichroic mirrors. These optical elements are responsible for redirecting the beams through the optical layout of the microscope and for combining the available excitation wavelengths into a common optical path before they reach the scanner.

The main objective of this subsystem is to ensure that the beam enters the scanner input aperture at a precise height of 7.4 cm above the optical table and propagates parallel to the table surface. To achieve this, the laser beam is first directed towards a pair of broadband dielectric mirrors: the first redirects the outgoing beam and lowers it to the required height, whereas the second adjusts the propagation direction such that the beam remains parallel to the optical table. This mirror pair is highlighted by the shaded region in Figure 2.6.

Following the initial mirrors, the beams are sequentially combined using a series of long-pass dichroic mirrors, each reflecting its respective beam into the common beam path while transmitting the others, thereby producing a single collinear optical path shared by all lasers. This alignment strategy ensures that all excitation wavelengths reach the scanner under the same geometric conditions, thereby preserving a common downstream optical path for light sheet generation.

System alignment is critical, as any angular deviation or focal shift introduced at this stage propagates through the illumination path and compromises light sheet formation and illumination uniformity. Once combined into a single collinear path, the beam is delivered to the scanning subsystem, where it is angularly deflected before entering the remaining illumination optics.

To facilitate the alignment process, each mirror was positioned so as to redirect the beam by 90° . Alignment was initiated with the 488 nm beam, which was used as the reference

beam owing to its frequent use, and the remaining lasers were then aligned sequentially. The beam height was adjusted to 7.4 cm by taking measurements at multiple points along the optical path using a ruler and by confirming parallelism with the optical table. The nominal 90° reflections were assessed using the optical table grid, and proper reflection alignment was then confirmed by placing two reference plates on opposite sides of a hole in the optical table and verifying that the beam passed between them. A third plate placed further downstream was used to confirm continued propagation along the intended optical path.

2.2.2 Illumination Path

After leaving the reflection optics, the combined excitation beam is delivered to the illumination path, whose function is to relay the collimated excitation beam and convert it into a dynamically scanned light sheet that selectively illuminates the sample. First, the beam reaches the scanner aperture, where it is deflected by a pair of orthogonally mounted galvanometric mirrors. In this setup, each mirror independently controls one axis (Y and Z). Only the Z -axis mirror is actuated to laterally scan the beam across the FOV, while the Y -axis mirror remains fixed, thereby ensuring beam stability along the orthogonal axis. As noted in Subsection 2.2.1, the beam reaches the scanner at a height of 7.4 cm. The scanner's original mechanical design does not match this height, and a custom scanner holder is therefore used to position it appropriately, as illustrated in Figure 2.7, such that the desired output height of 6.4 cm is obtained.

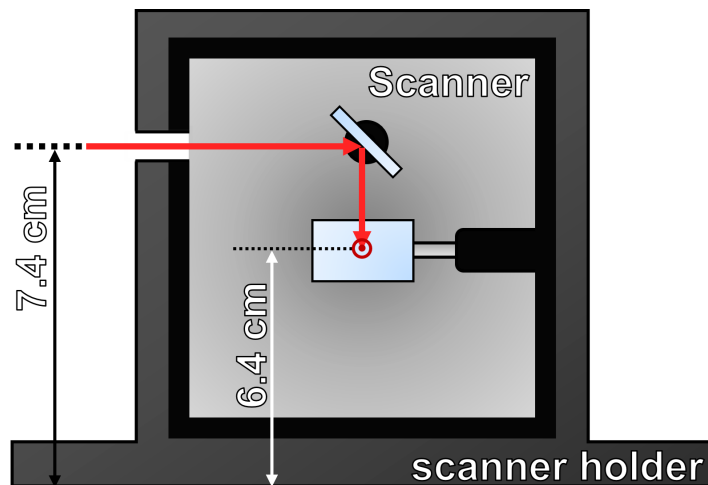


FIGURE 2.7: Illustration of the custom scanner holder (from Ref. [70]).

After leaving the scanner, the beam is directed towards the sample region through a modified illumination path, in which the Ronar 160 mm/532 nm f-theta lens previously used in

the system [70] was replaced by a Thorlabs TTL200-A tube lens [71]. This modification was introduced because the non-telecentricity of the f-theta lens prevented the beam from exhibiting the approximately parallel displacement at the image plane required for proper light sheet formation, particularly under rolling-shutter operation. As a result, the current illumination path includes two tube lenses in sequence, hereafter referred to as TL1 and TL2, as shown in Figure 2.8.

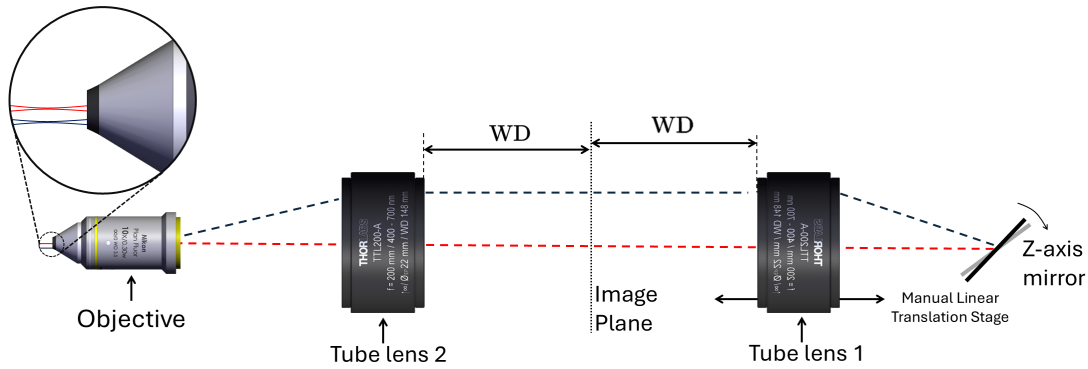


FIGURE 2.8: Schematic illustration of the illumination path of the light sheet microscope. Motion of the Z-mirror produces angular displacements in the input beam, which are subsequently corrected and focused at the back aperture of the objective by two tube lenses, thereby forming a thin light sheet at the sample plane. TL1 is mounted on a translation stage that allows adjustment of beam parallelism across the FOV (adapted from Ref. [70]).

Ideally, the two-lens relay system should satisfy a symmetric spacing condition: the distance between the scanner and TL1 should be equal to the distance between TL2 and the illumination objective, while the separation between TL1 and TL2 should correspond to twice the working distance ($2WD$) of the tube lenses, which are identical. In practice, however, the entrance pupil distance is defined over a range rather than as a fixed value [71]. Therefore, the distances between TL1 and the scanner, and between TL2 and the illumination objective, were adjusted experimentally in order to optimise the focus at the sample plane.

In addition, to optimise beam behaviour, TL1 was mounted on a manual linear translation stage, enabling fine adjustment of its position relative to the scanner and, consequently, to TL2. This additional degree of freedom allowed correction of the effective tilt of the scanned beam during scanner motion, thereby improving beam parallelism across the

FOV. In practice, controlled deviations were intentionally introduced at this stage in order to compensate for downstream spacing errors that could not otherwise be corrected. Although such deviations would nominally represent alignment errors, their compensatory effect improved the optical performance at the illumination objective and restored the desired beam propagation in the sample region.

In addition to the translation stage, the optomechanical mount of TL1 provides a further alignment degree of freedom, which is used to correct residual beam tilt and ensure that the beam propagates correctly through TL1 and TL2, exhibiting the desired behaviour at the sample plane.

After passing through TL1 and TL2, the scanned beam enters the illumination objective, which focuses the excitation light onto the sample and enables light sheet generation. TL2 and the objective are coupled within a 16 mm optical cage system that provides mechanical stability. The objective is mounted at the top of a translation optic mount, which is used to centre the Gaussian beam within the camera FOV. As shown in Figure 2.9, the system is mounted on an optical rail that allows alignment and adjustment during assembly procedures performed on the microscope. When positioned on the rail, the optical axis of the tube lens is located at a height of 6.4 cm above the optical table, thereby justifying the scanner holder and the height values adopted throughout the previous stages of the optical path.

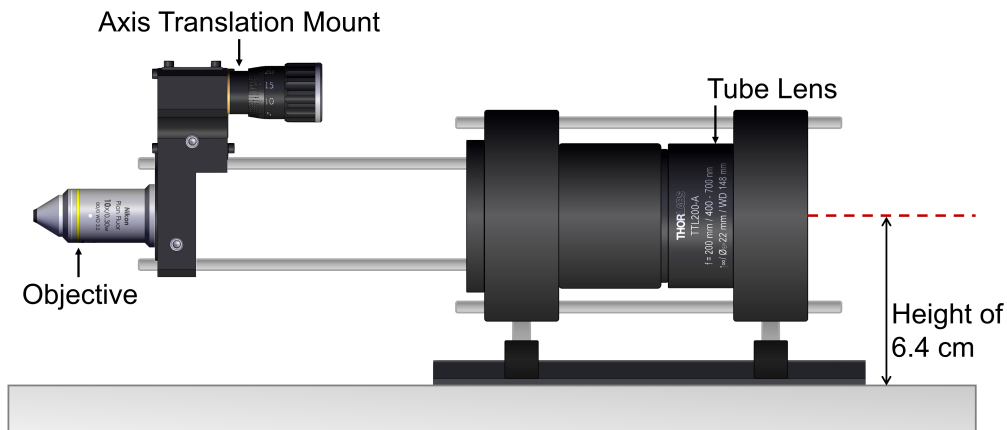


FIGURE 2.9: Illustration of the optical cage system installed on the light sheet microscope (from Ref. [70]).

2.2.3 Detection Path and Image Acquisition

After passing through the illumination path, the generated LS reaches the sample region. The sample is positioned within an immersion chamber, which defines the physical interface between the illumination and detection subsystems, as shown in Figure 2.10a. In this region, the scanned light sheet intersects the sample, generating fluorescence that is subsequently collected by the detection system. In this configuration, the definition of a coordinate system for sample motion is critical for precise positioning. Accordingly, the illumination plane generated by the scanner spans the XZ plane. The detection axis is orthogonal to this plane; therefore, the acquisition direction lies along the Y axis. This configuration justifies the use of the term Y -stack for volumetric data acquired by translating the sample along the Y axis while capturing sequential planes (Figure 2.10b).

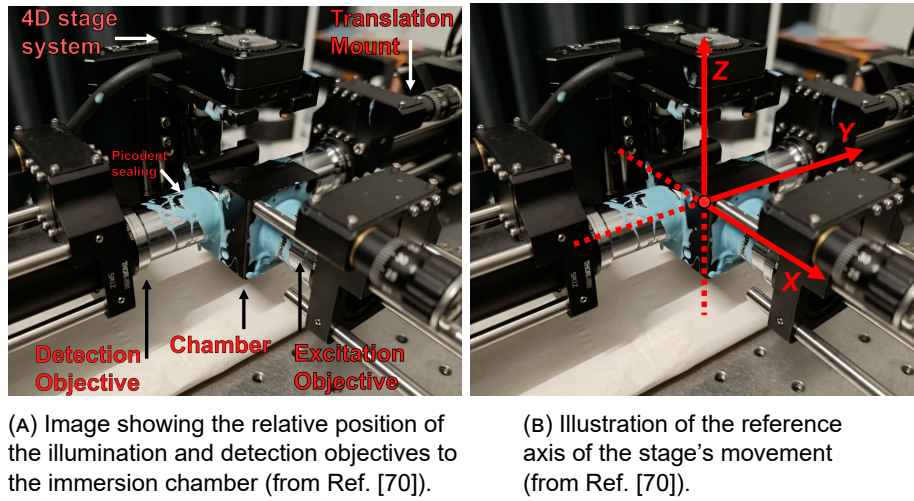


FIGURE 2.10: Immersion chamber used on the light sheet microscope. In (a), the design of the light sheet microscope is shown: single-sided illumination with two-sided detection. Focus adjustment is achieved by translating the mount present on all objectives. The chamber is sealed using Picodent TwinSil (cat. nb. 1300 1000). (b) Representation of the three reference axes in relation to image acquisition.

The fluorescence emitted by the sample is collected through a detection path that follows a standard infinity-corrected optical design. In this configuration, fluorescence emitted from the illuminated focal plane is collected by the objective and transformed into a collimated beam, with each object point corresponding to a specific propagation angle. The beam then propagates through a TTL200-A tube lens [71], which refocuses the collimated beam to form an image at the sensor plane. Accordingly, the sensor of the Hamamatsu ORCA-Flash4.0 V3 C13440-20CU sCMOS camera (Subsection 2.1.5) is positioned at a distance from the tube lens corresponding to its Working Distance (WD), thereby maximising image

focus and resolution at the sensor. A schematic of this detection pathway is shown in Figure 2.11.

This configuration is implemented in both detection arms, with a filter wheel (Subsection 2.1.4) positioned between the tube lens and the camera.

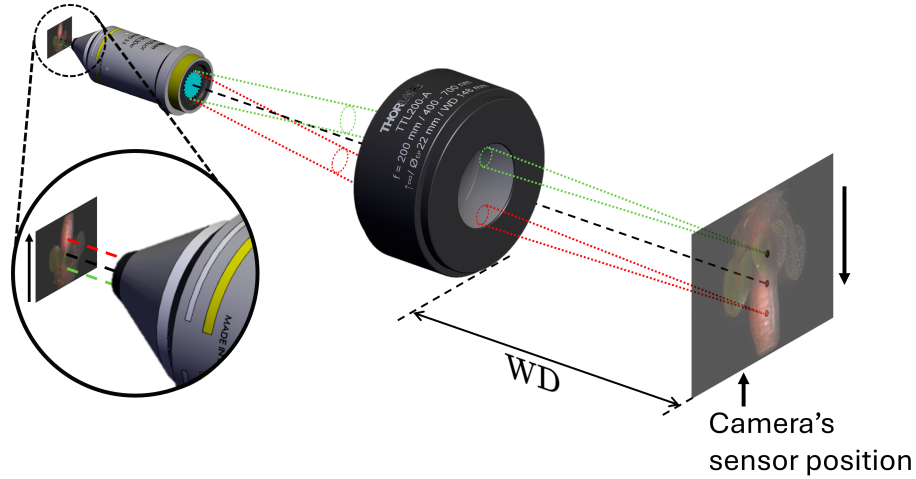


FIGURE 2.11: Detection pathway implemented in the light sheet microscope. Fluorescence emitted by the sample at the focal plane of the objective is collected and converted into collimated rays, each propagating at a distinct angle. These rays are then refocused by the tube lens to form a magnified image 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 originating from the sample are directed by the lens to specific positions in the image (from Ref. [70]).

As in the illumination path subsystem (Subsection 2.2.2), the objective and the tube lens are mounted within a 16 mm optical cage system. Likewise, this assembly is positioned on top of an optical rail to facilitate adjustments during microscope assembly. The objective is also connected to an axial translation mount, allowing image focusing and centring of the fluorescence emission within the FOV.

In terms of spatial resolution, the effective pixel size at the sample plane was experimentally estimated to be approximately $0.66 \mu\text{m}$ [70]. This value is in close agreement with the expected theoretical value of $0.65 \mu\text{m}$, taking into account the overall magnification of the detection optical system, including an effective $10\times$ scaling factor. The close correspondence between measured and theoretical values confirms the accuracy of the system's spatial calibration.

2.3. Software Architecture

The software architecture of the ALM LSFM provides a unified framework for hardware control, image acquisition and visualisation, as well as quality-control and error-management functionalities. The baseline implementation was developed by Simão Seixas in his work [70], and the current version builds upon it through the introduction of new components developed in this work, which are marked with an asterisk in Figure 2.12. These components include a mode switching function, which enables the system to switch between image acquisition mode and quality-control mode; a logging system, which allows error detection and monitoring of software and hardware events (its implementation is described in Chapter 5); a confocal acquisition mode synchronised with rolling shutter (described in Chapter 3); and an integrated quality-control system for the LS beam (described in Chapter 4). Full software code is available in Appendix G.1.

As shown in Figure 2.12, the software is organised into three main layers: the front-end graphical user interface, the control logic layer, and the back-end hardware communication layer. The front-end exposes the available microscope functions to the user through dedicated widgets, the control logic layer coordinates device behaviour and workflow logic, and the back-end layer provides low-level communication with the physical devices.

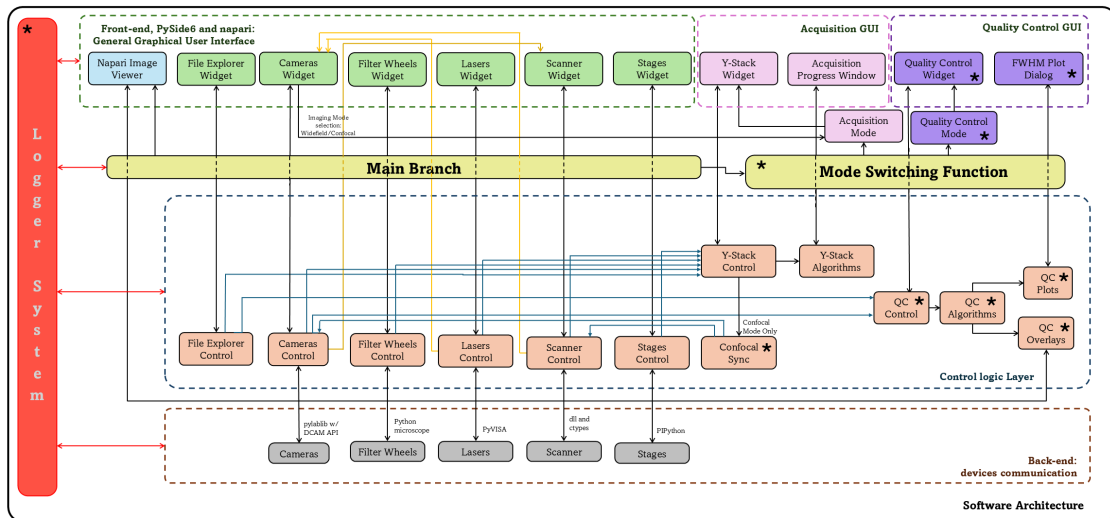


FIGURE 2.12: Overview of the current software architecture used to control the light sheet microscope. The system is organised into a front-end graphical interface layer, a control logic layer, and a back-end hardware communication layer. The scheme highlights the software components introduced in this work with an asterisk (*), namely the logger system, the confocal synchronisation module, and the quality-control analysis and visualisation modules (adapted from Ref. [70]).

2.3.1 Python as the selected Programming Language

The software was developed entirely in Python, which serves as the primary programming language for hardware control, user-interface implementation, synchronisation between system components, and error tracking. Python was selected owing to its flexibility, readability, and extensive ecosystem of scientific libraries, together with packages tailored to instrumentation and microscopy, thereby enabling communication between microscope components, acquisition logic, image visualisation, quality-control analysis, system monitoring and error logging to be integrated within a single software environment. Moreover, its community-driven development model and widespread adoption within academia contribute to its long-term maintainability and continued evolution.

This choice also supports the modular, layered software architecture shown in Figure 2.12, in which the back-end for device communication, the control logic layer, and the front-end are clearly separated. Within this framework, the front-end is implemented using napari and PySide6. napari is used as the image viewer and is represented in blue in the figure, whereas PySide6 is used to implement the GUI widgets. In the diagram, general-purpose widgets are shown in green, the Acquisition Mode widgets are shown in pink, and the Quality Control Mode widgets are shown in purple.

At the back-end layer, Python interfaces directly with the microscope hardware through device-specific libraries. These include PyLabLib in combination with the DCAM-API for camera control, Python Microscope for communication with the filter wheels, PyVISA for laser control, DLLs accessed via `ctypes` for scanner communication, and PI Python for stage control. By isolating these dependencies within a dedicated hardware communication layer, the software architecture decouples device-specific communication from the higher-level system logic.

This work introduced the implementation of an important logging system that spans the entire software stack. As shown in Figure 2.12, data logging acts as a cross-cutting component across the GUI, the control logic, and the hardware communication layers. Its purpose is to record microscope component states, software events, acquisition and control parameters, and error conditions in a consistent and traceable manner. This functionality improves system robustness and facilitates both error debugging and the reproducibility of microscope operation. In addition to the logger system, Python was also used in this work to implement the mode switching function, the confocal synchronisation module, and the quality-control workflow integrated into the software architecture.

2.3.2 napari as Image Viewer

napari is an open-source image viewer developed in Python, designed for high-dimensional microscopy data. Its live-image visualisation capabilities and support for multidimensional datasets (organised into planes, channels, and time points) make it particularly well suited to light sheet microscopy [72].

In the current implementation, napari is integrated directly into the graphical environment and constitutes one layer of the software interface. The viewer provides visual feedback during alignment, acquisition, and system validation, enabling the user to inspect the focus, FOV, signal intensity, and the image currently being acquired by the camera in real time.

In addition, napari is integrated into the QC workflow, where QC overlays for focus and light sheet alignment are displayed. Consequently, napari serves not only as a camera image viewer, but also as an integral component of the software environment for the visual and quantitative evaluation of microscope performance.

2.3.3 OME-Zarr for Image Storage

For the storage of acquired image data and associated metadata, the ALM software adopts the OME-Zarr format. This format is particularly well suited to LS microscopy, as it supports high-resolution multidimensional datasets together with structured metadata, enabling both image data and acquisition parameters to be stored within a unified file organisation [52, 73–75]. It is readily accessible, for example, in Fiji [76], a widely used image analysis platform, including among ALM users.

2.3.4 Users Interaction

Upon launching the software, the user is given the option to select a directory on the control computer in which all files generated during operation are to be stored. This selection may be modified after proper initialisation of the software.

Once the initialisation of the different devices is complete, the full GUI in acquisition mode is ready and displayed on screen, as shown in Figure 2.13. The GUI provides a unified environment in which cameras, lasers, the scanner, the stage, filter wheels, and acquisition or control tools, depending on the selected mode, are arranged on the left-hand side of the main window in dedicated widgets, while the napari image viewer occupies the

right-hand side, forming a single integrated interface. A background thread continuously monitors the camera buffers for new frames and forwards them to napari in real time, with images from each camera assigned to separate layers within the viewer.

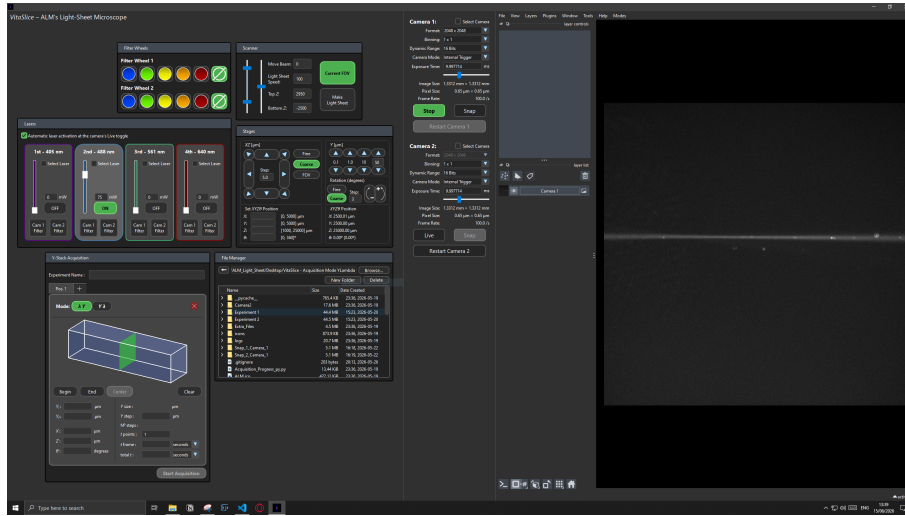


FIGURE 2.13: Main GUI of the custom-developed software, showing all control panels for the acquisition mode and a real-time image of a light sheet beam from Camera 1. The software provides control over each microscope device, including lasers, filters, the scanner, stages, and cameras, as well as control of the operating modes (acquisition mode and QC mode).

A key contribution of this work is the introduction of the mode switching function, which enables switching between Acquisition Mode and Quality Control Mode within the software environment (Figure 2.14a). In Acquisition Mode (shown in Figure 2.13), the software presents the Y-Stack Acquisition Widget, which allows the user to define stage motion and time points, as well as the dedicated widgets for selection of hardware settings for the camera, laser excitation, scanner operation, and filter wheel. Once the desired parameters are defined, the acquisition sequence is executed with real-time feedback provided by napari, while acquisition progress is displayed in the corresponding control windows.

In addition to enabling acquisition in widefield mode, this work introduces a confocal mode based on synchronisation between the scanner and the camera rolling shutter. These modes can be selected within the corresponding camera widget, as shown in Figure 2.14b.

In Quality Control Mode, the acquisition widget is replaced by the QC panel (Figure 4.1b). Through this panel, and the associated analysis modules described in Chapter 4, the software evaluates beam quality in real time using overlays in napari image of the light sheet position and focus, together with quantitative metrics such as beam angle and focus.

In addition, the FWHM of the beam is computed and visualised in a Matplotlib graph, with its value at focus also reported, and the possibility to generate and save a quality-control report. This integration enables the execution of QC procedures directly within the microscope software, without the need for external analysis tools.

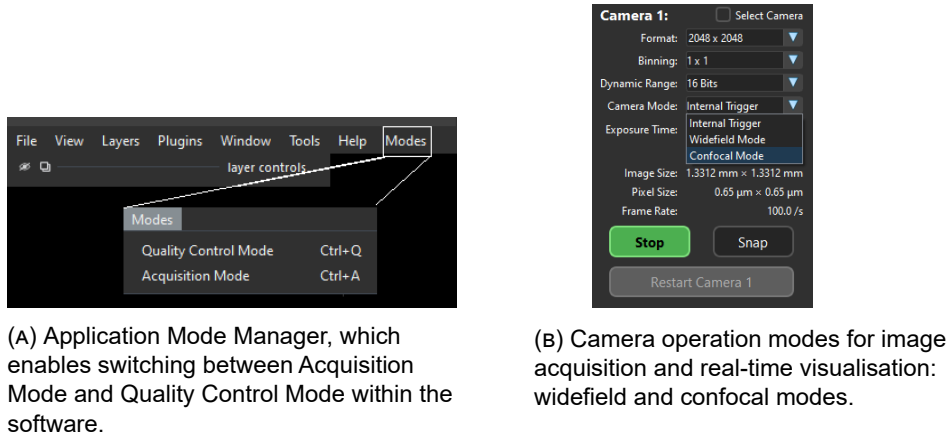


FIGURE 2.14: Overview of the different operating modes available in the software. (a) The software offers two working modes: the Quality Control Mode and the Acquisition Mode, selectable from the main tab on the napari window. (b) Switching between Confocal Mode and Widefield Mode is done directly on the camera widget, in the “camera mode” option.

Throughout software operation, the entire workflow is continuously logged, from device communication to program termination. The log file is saved as a .txt file in the logs directory within the user-selected output folder. This functionality enhances transparency and traceability during operation by continuously recording hardware states, software events, acquisition parameters, and error conditions, thereby supporting debugging, system monitoring, and reproducible operation of the ALM microscope software.

3. Rolling Shutter Implementation

In the software originally controlling the ALM microscope, a Global Shutter acquisition configuration was set as default. In this implementation, the software already supported coordinated hardware control, volumetric acquisition, and real-time image visualisation; however, a synchronisation strategy between scanner motion and the camera rolling shutter had not yet been implemented. This approach had already been identified in the previous work as a further development to be pursued, which ultimately motivated the work presented in this chapter [70].

3.1. Global Shutter as Widefield Mode

As explained in Subsection 1.3.2, in Global Shutter mode the camera integrates the fluorescence signal over the entire sensor. There is no confinement of the detection region to the instantaneous position of the light sheet.

From a software perspective, widefield mode is based on the detection by the camera sensor of fluorescence generated by the light sheet, which is formed through continuous scanning of the laser beam using the scanner's galvanometric mirror. In the implementation previously developed [70], real-time visualisation of the camera image in this mode is achieved through a repetitive vertical sweep of the beam across the camera FOV, from top to bottom, while the camera continuously acquires images. In this configuration, the scanner acts as the master thread, initiating the sweep, while the camera remains active throughout the scanning cycle until its completion. The laser emission remains continuously on during the entire scanning process and returns to its initial position at the end of each cycle.

Following the modifications introduced in the illumination path described in Subsection 2.2.2, an artefact became apparent in images acquired in widefield mode during real-time visualisation outside acquisition sequences. This artefact, characterised by the presence of a high-intensity band located at the top of the image, while the rest of the image showed a homogeneous illumination, is shown in Figure 3.1a. The increased intensity observed in the upper region indicates the presence of unwanted additional illumination in that portion of the sensor during Global Shutter operation.

Since the laser remained active not only during the forward scanning sweep that generates the LS, but also during the return motion of the mirror to its initial sweep position, fluorescence was detected by the sensor outside the intended imaging phase, leading to the accumulation of unwanted signal in the region corresponding to the beginning of the sweep.

To eliminate this effect, the laser control logic was modified so that emission occurs exclusively during the top-to-bottom sweep responsible for generating the LS. Consequently, the laser is switched off during the return motion to the initial sweep position. This modification ensures that laser emission is confined to the time interval during which the camera is acquiring the image, thereby preventing the accumulation of signal outside the intended LS trajectory.

The modifications to the scanning behaviour were implemented within the scanner control workflow, which executes continuous LS scanning through dedicated worker threads. Laser control is handled through the RTC5 board operating in EXTERNAL DIGITAL, ensuring that the excitation lasers are active only during the forward scanning phase responsible for image formation.

It is important to note that, during real-time visualisation in Widefield mode outside acquisition, an additional dwell period of 10 ms was introduced at the end of each scan. This interval was sufficient to ensure that the laser was fully disabled before the scanner returned to the top of the FOV. During this dwell, the laser remains switched off, preventing the residual illumination that would otherwise produce a visible beam trace at the bottom of the image. In contrast, during acquisition mode, this additional dwell is not required. This is because each frame acquisition inherently includes delays associated with hardware synchronisation and image transfer to the camera memory, which effectively prevent the accumulation of unintended signal.

As a result of these modifications, the illumination behaviour was corrected, leading to the effective removal of the artefact and to a uniform intensity distribution across the FOV, as shown in Figure 3.1b.

Overall, this modification highlights the importance of explicitly controlling laser emission during non-imaging phases of the scanning cycle, even in widefield acquisition modes.

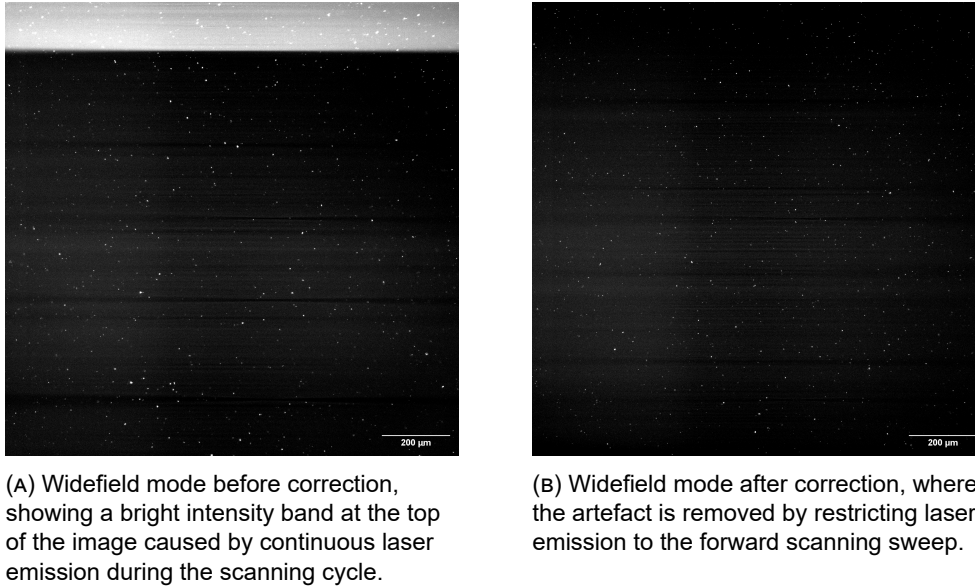


FIGURE 3.1: Effect of laser-scan synchronisation in Widefield mode with water in the chamber: before (a) and after (b) correction.

3.2. Rolling Shutter as Confocal Mode

One of the main contributions of this work is the implementation of a confocal acquisition mode based on the synchronisation between the scanner and the camera readout in rolling-shutter mode. This approach introduces temporal and spatial coupling between illumination and detection, confining the detected signal to a narrow region that follows the excitation beam. Consequently, improved optical sectioning is achieved, along with reduced background and enhanced SNR compared to the Global Shutter configuration described in Section 3.1.

3.2.1 Principle of Operation

The Rolling Shutter mode, referred to in the software as confocal mode (Figure 2.14b), relies on precise synchronisation between the sweeping of the LS beam generated by the scanner and the line-by-line sequential readout of the camera sensor. Unlike widefield mode, the detector does not expose all sensor lines simultaneously; instead, each subset of lines is exposed at different times. By aligning the instantaneous position of the beam with the active readout region of the sensor, the system effectively acts as a virtual slit that follows the LS across the FOV.

In the LSFM system at ALM, the Hamamatsu ORCA-Flash4.0 V3 camera [67–69] operates in this mode using the *Lightsheet Readout Mode*, which has been specifically designed for synchronisation between sensor readout and the scanning of a light sheet. In

this configuration, the detector is read from top to bottom, corresponding to the direction of beam propagation during the scan.

3.2.2 Master–Slave Synchronisation Strategy

In widefield mode, the scanner acts as the master in the synchronisation process. For the confocal mode, an alternative approach initially considered was to use the camera as the master and the scanner as the slave through temporal control based on an *HSYNC*-type signal. However, the scanner control board (RTC5) no longer provided the available resources required for this synchronisation scheme, and modifying the master–slave relationship in one mode would have implied altering it in widefield mode as well, which was not desirable.

Consequently, the scanner was retained as the master, and the camera operates in *Light-sheet Readout Mode*, in which detector readout follows the timing imposed by the scanner sweep through a calibration procedure described in Subsection 3.2.4. This approach ensures that the galvanometric scan remains the reference signal throughout the entire acquisition in both modes and is consistent with the control architecture described in Chapter 2.

3.2.3 Exposure Time Constraints

For beam calibration during rolling-shutter operation, it is important to determine the limits of the camera exposure time and, consequently, the limits of the scanner sweep speed required to maintain synchronisation. The exposure time is constrained by the sensor readout mechanism in *Lightsheet Readout Mode*. As defined in the Hamamatsu ORCA-Flash4.0 V3 manual [69], the valid exposure time is determined by the line readout time $1H$ and the full-frame readout time $V_n \cdot 1H$, where V_n is the number of vertical lines.

For full-frame operation, the camera has 2048×2048 pixels, and therefore $V_n = 2048$. Given that $1H \approx 9.744 \mu\text{s}$, this yields a maximum effective exposure time of approximately 19.96 ms . This value has been verified experimentally, with the exposure time not exceeding this upper limit.

Although the camera hardware supports a wider range of exposure times, the usable exposure interval for synchronised rolling-shutter operation at 2048×2048 pixels lies approximately within the range $9.7 \mu\text{s}$ to $19\,956 \mu\text{s}$.

3.2.4 Experimental Calibration of Exposure Time and Scanner Speed

Reliable synchronisation between the rolling-shutter readout and the scanning beam is essential for stable operation and high-quality image acquisition in confocal mode. For this reason, a direct relationship between the camera exposure time and the scanner speed was determined experimentally.

For each selected exposure time, the scanner speed was manually adjusted in order to identify the minimum velocity that allowed the scanned beam to correctly fill the camera FOV. The resulting exposure–speed pairs were then used to define the operating range of the synchronised confocal mode. The measured data exhibit the expected inverse relationship: shorter exposure times require higher scanner velocities so that the beam can cover the FOV within the available readout interval. The complete set of experimentally determined exposure–speed pairs used for confocal synchronisation is presented in Appendix D.

The experimentally determined relationship was subsequently incorporated into the software through the calibration model that provided the best fit to the measured data. In the present implementation, this corresponded to a quadratic fit, as illustrated in appendix Figures D.1a and D.1b. It provides stable and reproducible behaviour during operation of the confocal rolling-shutter mode.

However, this empirical relation does not currently admit a direct physical description of scanner–camera synchronisation. For this reason, a more physically grounded model is under development. As it is not yet fully operational, it has not been implemented; however, it provides a theoretical framework for understanding the relationship between exposure and the measured scanning speed.

The model is based on a linear mapping between the top and bottom scanner coordinates (s) and the camera pixel coordinates (p_n):

$$p_n = m \cdot s + b \quad (3.1)$$

Since the detector has 2048×2048 pixels, the pixel coordinate range extends from 0 to 2047. The scanner covers a range from -2900 to 2300 scanning units to span the entire FOV of the camera. From this, the parameters are estimated as $m = \frac{2047}{2300 - (-2900)} \approx 0.394$ and $b = 1142$, yielding the relation $p_n \approx 0.394 \cdot s + 1142$.

Using this mapping, the scanner velocity required to traverse the full FOV within an exposure time t_{exp} is given by $v_{\text{scanner}} = \frac{\Delta s}{t_{\text{exp}}}$, where t_{exp} is expressed in milliseconds and v_{scanner} in scanner units per millisecond. In the camera domain, this relationship can also be expressed as $v_{\text{scanner}} = \frac{\Delta p}{m \cdot t_{\text{exp}}}$.

For this reason, the physical model is currently regarded as an interpretative and optimisation framework rather than as the operational synchronisation model.

3.2.5 Software Implementation

At code level, the synchronisation logic between camera exposure time and scanner speed is implemented through the functions defined by Equations D.1 and D.2 (Appendix D), together with limiting functions that restrict operation to the validated synchronisation limits. These constraints ensure that the system remains within the regime in which the scanned beam correctly fills the FOV and the rolling-shutter acquisition remains stable.

Within the scanner-control architecture, selection of the confocal mode through the camera widget activates a dedicated `RollingShutterWorker`, which is responsible for maintaining the synchronised sweep required for rolling-shutter acquisition. This distinguishes the confocal mode from the widefield mode at the software level, since each mode follows a separate execution pathway within the scanner workflow.

As in Widefield mode (Section 3.1), laser emission is confined to the effective imaging phase of the scan. In confocal mode, the laser remains active only during the forward top-to-bottom sweep that is synchronised with the rolling-shutter readout, and is switched off during the return motion to the top of the FOV. This prevents fluorescence generated outside the valid synchronisation interval from contributing to the acquired image.

3.2.6 Step-by-Step Acquisition Workflow

From both the user and software perspectives, the confocal rolling-shutter mode follows a structured acquisition sequence, as illustrated in Figure 3.2.

When operating either in confocal acquisition mode or in live image visualisation, the user activates Confocal Mode through the camera widget. At this stage, the user may define either the desired camera exposure time in the camera settings or the scanner speed in the corresponding widget. The software then computes the corresponding synchronisation parameter through the functions described in Subsection 3.2.4.

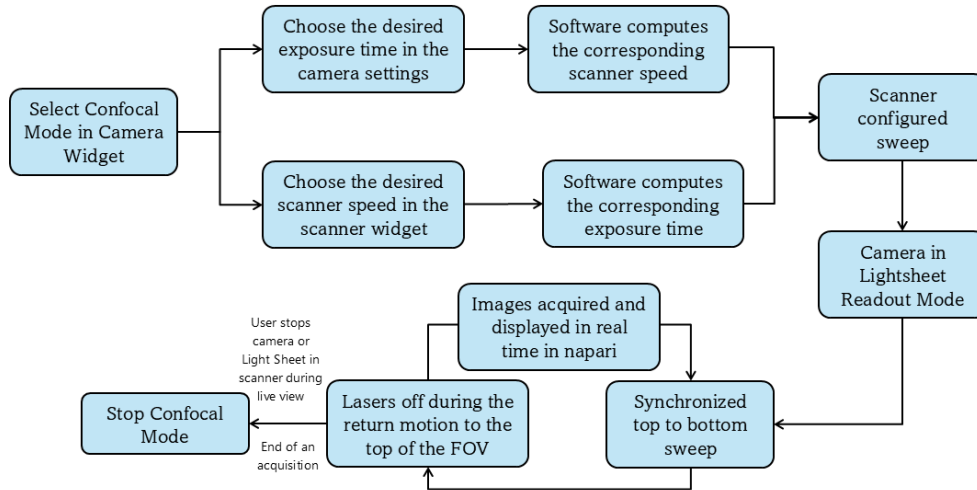


FIGURE 3.2: Software workflow of the confocal rolling-shutter mode. The user first activates Confocal Mode and then defines either the desired camera exposure time or the scanner speed. The software computes the corresponding synchronisation parameters, configures the scanner sweep, operates the camera in *LightSheet Readout Mode*, and executes the synchronised scan through the *RollingShutterWorker*. During the forward top-to-bottom sweep the excitation lasers remain active, while during the return motion to the top of the FOV they are switched off. The acquired images are displayed in real time in napari.

The acquired images are displayed in real time in the napari viewer, enabling the user to monitor focus, signal intensity, and correct synchronisation behaviour throughout the operation. The mode can be terminated either by the user or at the end of an acquisition sequence or during live operation by pressing the stop control on the camera or disabling the Light Sheet on the scanner via the interface.

3.2.7 Validation Strategy and Discussion

At the current stage of development, the confocal mode with rolling shutter operates using an experimentally derived calibration based on a quadratic fit. The physically motivated model described in Subsection 3.2.4 represents an important step towards optimising the calibration of this method. However, any validation of the approach must ultimately rely on experimental verification.

In addition to the uncertainty associated with the scanner units, deviations from the physical model may arise due to system-specific effects, such as control signal latency, which can effectively increase the exposure time, and potential non-linearities in the galvanometric response.

Overall, the confocal mode developed in this work represents a significant extension of the original acquisition framework implemented in the ALM microscope. Whereas the widefield mode relies on the integration of fluorescence over the entire detector, the confocal mode introduces explicit synchronisation between scanner motion and rolling-shutter readout. The benefits of this approach are demonstrated in the results presented in Section 3.3.

3.3. Differences between Widefield and Confocal

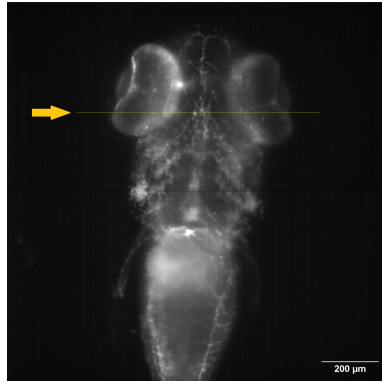
To evaluate the practical impact of implementing a confocal rolling-shutter mode relative to the widefield Global Shutter configuration, images were acquired in both modes across different sample types. In this work, the comparison is primarily based on zebrafish samples (Subsection 3.3.1) and hydrogels (Subsection 3.3.2), where clear visual differences between the two modes are observed. Additional comparison was performed on neural crest cell spheroid samples (Appendix E).

To ensure a rigorous and unbiased qualitative comparison, all images were processed using Fiji [76], with identical brightness and contrast settings applied within each pairwise comparison. Average Y projections were used for the neural crest cell spheroid, hydrogel, and blood vessels labelled zebrafish sample. For multi-channel datasets, the colour mapping associated with each excitation channel was preserved consistently across both acquisition modes.

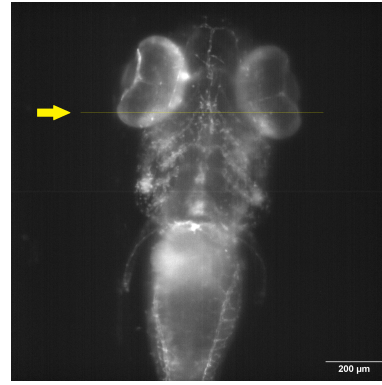
3.3.1 Labelled blood vessels Zebrafish Sample

This subsection presents a comparison of a zebrafish with labelled blood vessels acquired in confocal and widefield modes using an average-intensity Y projection, as shown in Figure 3.3a and 3.3b.

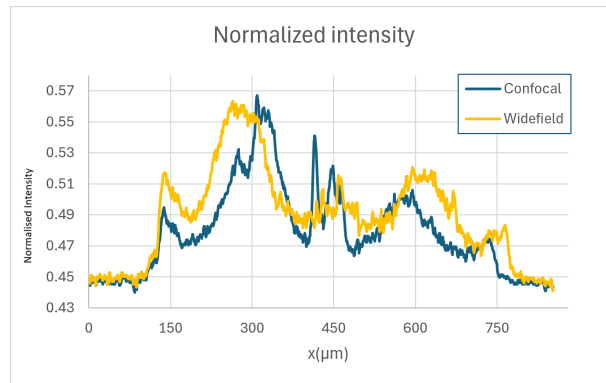
In the confocal mode image (Figure 3.3a), the background appears noticeably darker and the vascular structures are more clearly defined. In the widefield mode image (Figure 3.3b), the background is more diffuse, reducing the apparent separation between the vascular structures and the surrounding signal. This comparison shows that, for this type of sample, confocal mode provides a visually clearer representation of the labelled structures than widefield mode.



(A) Zebrafish sample with labelled blood vessels acquired in Confocal Mode. The yellow line and arrow indicate the region from which the intensity values extracted.



(B) Zebrafish sample with labelled blood vessels acquired in Widefield Mode. The yellow line and arrow indicate the region from which the intensity values were extracted.



(c) Normalised intensity along the yellow line region in (a) and (b) highlighted by the arrow.

FIGURE 3.3: (a,b) Comparison between widefield and confocal acquisition for a blood vessels labelled zebrafish sample using average-intensity Y projections. Both images were processed in Fiji using the same brightness and contrast settings. The Confocal projection exhibits a darker background and improved definition of the labelled vascular structures relative to the Widefield projection, which shows a more diffuse background contribution. c) Normalized intensity along the yellow line in (a) and (b). Confocal data is represented in blue, whereas the widefield data is represented in yellow.

In the confocal mode image, the background appears noticeably darker and the vascular structures are more clearly defined. In the widefield mode image, the background is more diffuse, reducing the apparent separation between the vascular structures and the surrounding signal. This comparison shows that, for this type of sample, confocal mode provides a visually clearer representation of the labelled structures than widefield mode.

The normalised intensity profile, calculated as $I_{\text{norm}} = \frac{I}{(I_{\text{max}} + I_{\text{min}})}$, is shown in Figure 3.3c supports this qualitative observation. Along the selected line, the confocal profile exhibits sharper intensity transitions with a higher peak values compared to widefield mode. A lower background is also evident in the images acquired with confocal mode relative

to widefield. This analysis is consistent with the results reported by Baumgart and Kubitcheck, where intensity profiles were used to compare global shutter and rolling shutter detection, showing improved contrast and lower background in rolling-shutter-based confocal images [77].

3.3.2 Hydrogel Sample

A further comparison was performed using a hydrogel sample with astrocyte cells, as shown in Figure 3.4. Here, different markers were used to label the cells, and the fluorescence channels are represented consistently across both acquisition modes: green corresponds to the 405 nm excitation beam, cyan to 488 nm, magenta to 561 nm, and grey to 640 nm. Labelled astrocyte cells are visible across the hydrogel, and morphological details are clear, such as the presence of processes in some of the cells. In the widefield acquisition (Figure 3.4b), a diffuse background signal is present in the image, which impairs the contrast and the ability to distinguish the fine processes of the cells, these being the finest details to quantify in such a sample. This clearly demonstrates the capacity of the confocal mode to reject background fluorescence while preserving local structural details (Figure 3.4a).

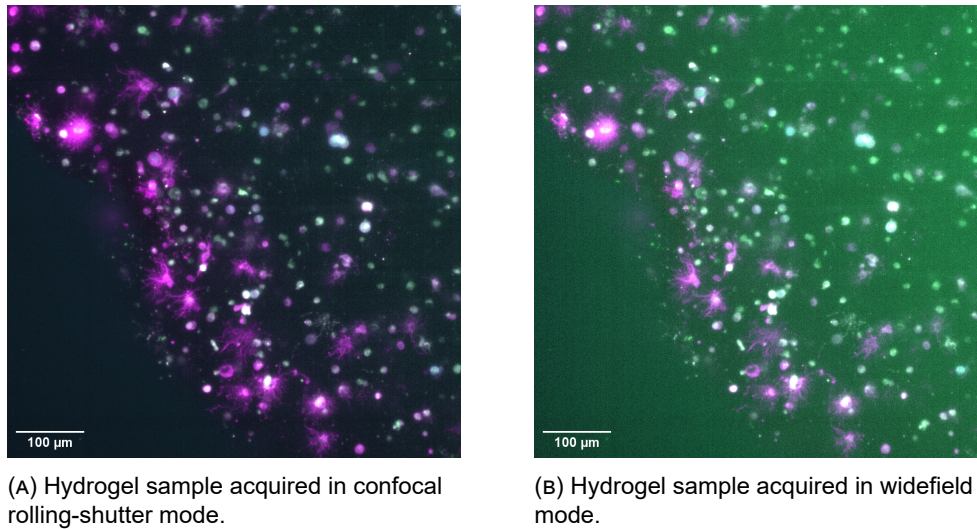


FIGURE 3.4: Qualitative comparison between confocal rolling shutter mode (a) and widefield mode (b) for a hydrogel containing astrocyte cells. The confocal image shows reduced diffuse background and clearly resolved cell processes relative to the widefield image. Both images use the same brightness and contrast settings and the same colour assignment for all channels (green = 405 nm, cyan = 488 nm, magenta = 561 nm, grey = 640 nm.).

The comparison between the two modes using the hydrogel sample reinforces the conclusions drawn from the zebrafish analysis. Confocal-mode acquisition produces sharper

images with improved out-of-focus light rejection, whereas the widefield mode exhibits a higher contribution of unwanted signal, leading to reduced image contrast.

3.3.3 Overall Results

The comparisons performed using the blood-vessel-labelled zebrafish sample (Subsection 3.3.1) and hydrogels containing astrocyte cells (Subsection 3.3.2) indicate that the confocal rolling-shutter mode introduced in this dissertation provides a clear practical improvement over the widefield configuration. The use of rolling-shutter synchronisation reduces out-of-focus light contributions and enhances the perception of local structural details in the image, in agreement with the expected behaviour arising from synchronisation between the scanner and the camera.

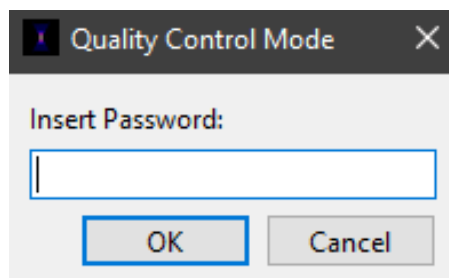
This result is consistent with previous work showing that synchronising a scanned light sheet with the rolling shutter of an sCMOS camera creates an effective moving confocal slit, reducing scattered and out-of-focus fluorescence relative to global-shutter detection [77]. Similar improvements in background rejection and image contrast have also been reported for electronic confocal slit detection in biological samples [36, 78].

4. ALM LSFM Characterisation

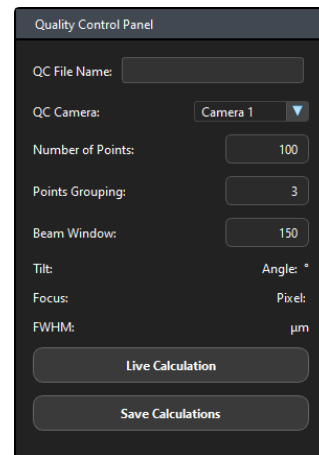
The characterisation of a light sheet microscope is essential for ensuring high-quality image acquisition. To this end, a QC methodology was implemented within the software to evaluate the optical performance of the LS and to provide quantitative feedback on beam alignment. Furthermore, bead-based evaluation methods were developed to assess system chromatic between excitation beams, together with the use of homogeneous fluorescent dye solutions to evaluate field uniformity. These combined approaches enable a comprehensive assessment of the overall performance of the microscope.

4.1. Quality Control Widget

As outlined in Section 2.3, a dedicated Quality Control mode was implemented within the ALM microscope software for beam characterisation. Access to this mode requires authentication via a password (Figure 4.1a), ensuring that it is restricted to authorised users. This mode is intended for use by ALM personnel for microscope evaluation and realignment. Once activated, the QC widget (Figure 4.1b) becomes accessible, enabling beam characterisation procedures to be conducted directly within the same software environment used for image acquisition and visualisation.



(A) Password dialog required to access the Quality Control mode, ensuring restricted access to authorised users.



(B) Quality Control Widget illustrating the configurable analysis parameters and available QC actions for beam characterisation and overall system evaluation.

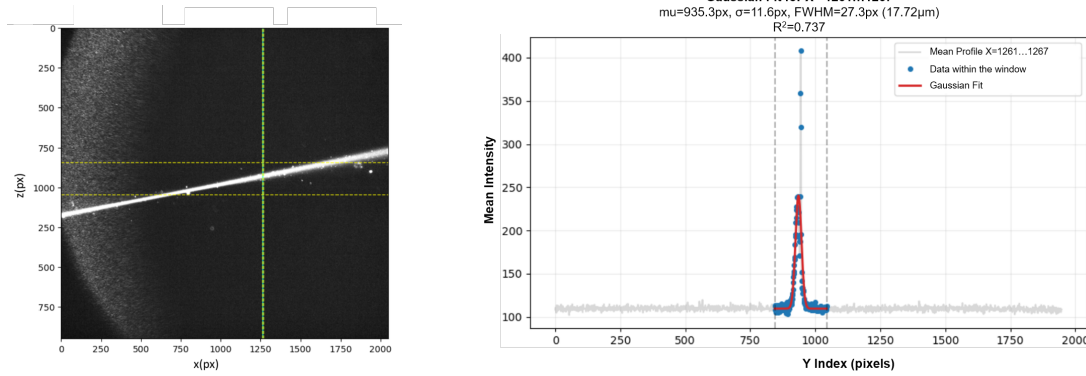
FIGURE 4.1: Quality Control interface of the ALM microscope software.

At the application level, the QC widget is integrated within the GUI, as shown in Figure 2.12. The widget enables numerical analysis, overlay visualisation in napari, and

graphical plotting within the software environment. Furthermore, it supports data export through the QC workflow, including the generation of PDF reports, .csv data files, image recordings with overlays, and plots saved in .png format.

To perform the calculations required for beam analysis, the widget includes a set of user-configurable parameters. As shown in Figure 4.1b, these comprise a file name parameter used to define the output folder within the file manager directory, the selection of one or both cameras for characterisation, the number of sampling points along the beam used in the analysis, the grouping of neighbouring points for local averaging, and the beam window employed for profile extraction. Together, these parameters determine the number of transverse beam sections analysed, the method of local averaging, and the size of the fitting region around the beam.

At the level of numerical beam analysis, the QC workflow is based on Gaussian fitting of beam profiles extracted from the most recently acquired camera frame, since the LS can be well approximated by a Gaussian intensity distribution. A series of equally spaced columns is selected across the image, with the spacing determined by the number of points defined for the calculation.



(A) Beam image with the selected column region used for profile extraction. The vertical green dashed line indicates the analysed column position, while the two dashed yellow horizontal lines define the fitting window around the beam.

(B) Extracted intensity profile and corresponding Gaussian fit within the window of 1261 to 1267 pixels. The grey curve represents the mean profile along the selected column range, the blue points correspond to the data within the fitting window, and the red curve represents the fitted Gaussian model.

FIGURE 4.2: Example of the Gaussian fitting procedure used for beam characterisation. A local intensity profile is extracted from a selected region of the beam image and fitted with a Gaussian model to determine the beam centre, baseline, standard deviation, FWHM, and coefficient of determination R^2 .

For each selected column, the corresponding intensity profile is extracted and fitted with a Gaussian model, from which the beam centre, baseline, standard deviation, full width

at half maximum (FWHM), and the coefficient of determination R^2 are obtained. Repeating this procedure for all selected columns allows the variation of beam width and beam position to be characterised across the FOV. An example of this procedure is shown in Figure 4.2. A selected column region is extracted from the beam image, and the corresponding transverse intensity profile is fitted with a Gaussian function to estimate the beam centre and width.

From these data, the beam tilt angle is estimated via a linear regression applied to the centres of the detected beam profiles across all analysed columns. Furthermore, the focus position and the corresponding FWHM are obtained from the coordinate of the minimum of a hyperbolic fit describing the evolution of the FWHM along the beam.

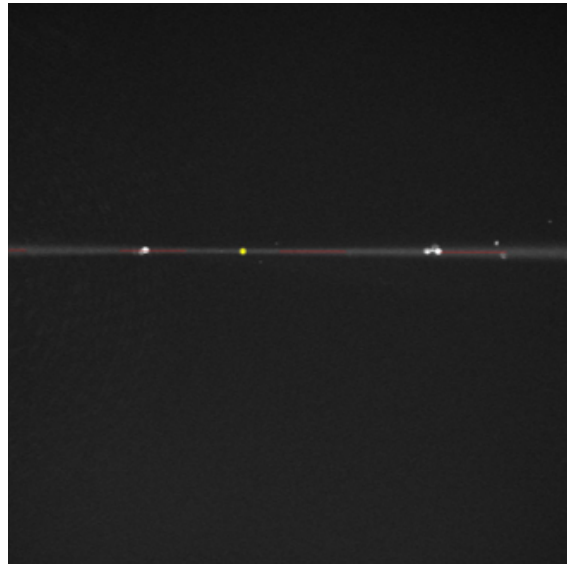


FIGURE 4.3: Image of the laser beam in water. The red lines in overlay are used to determine the beam tilt. The yellow dot marks the estimated beam focus position, where the beam waist is thinner.

For visual feedback within the application, the QC workflow is tightly integrated with the napari viewer, as shown in Figure 2.12. A red overlay line indicates the beam tilt, while a yellow marker denotes the estimated focus position (Figure 4.3).

These overlays are continuously updated with incoming image data, ensuring smooth operation without GUI freezing or instability. In addition, a PySide6 dialog incorporating a Matplotlib plot displays the FWHM profile along the beam, together with the corresponding hyperbolic fit and the estimated focus coordinate. This functionality is illustrated in Figure 4.4, showing the widget in active operation. This integrated presentation enables the user to simultaneously monitor the visual appearance of the beam and its quantitative characterisation within a unified software environment.

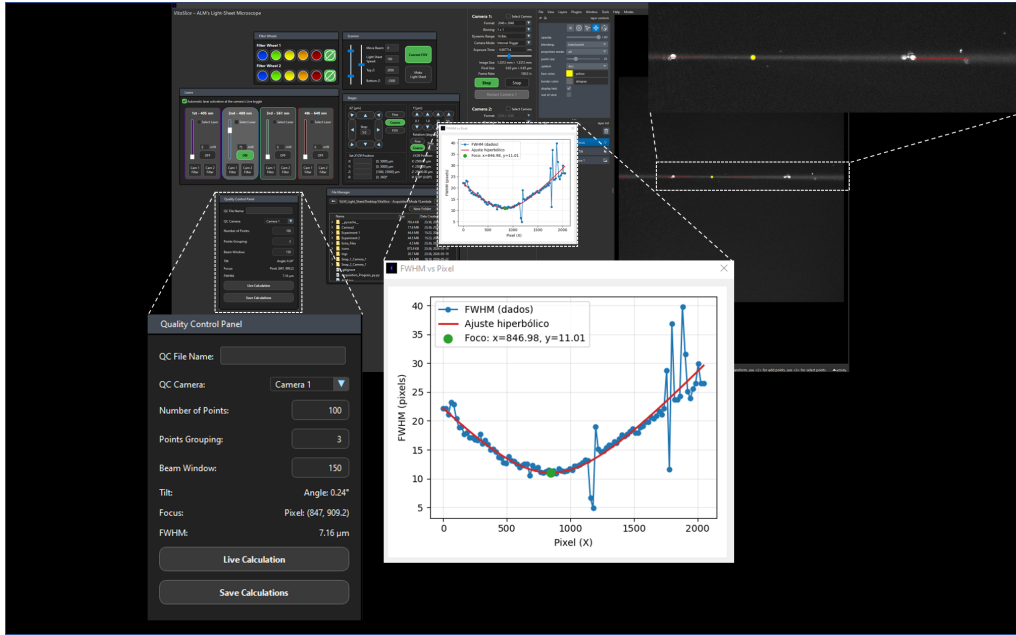


FIGURE 4.4: Quality Control Widget in operation. The napari viewer displays the analysed beam with overlays, while the FWHM plot dialog and the calculated QC parameters are shown simultaneously. In this example, calculated FWHM in focus has 11.01 pixels.

The widget provides two main modes of operation: *Live Calculation* and *Save Calculations*. The live mode is intended for immediate inspection and characterisation of the beam, supporting real-time alignment. This mode operates on a per-camera basis. The save mode records the results of the current analysis by generating a PDF report, a structured .csv data file, and images of the beam with overlays, together with the corresponding FWHM profile plot along the beam.

4.2. QC Report

As discussed in Section 4.1, in addition to real-time visualisation, the QC widget enables the storage of a set of outputs that document both the numerical analysis and the corresponding visual information.

The QC report generated through the *Save Calculations* option is designed to provide a concise yet comprehensive summary of beam characterisation for each camera. The report includes the date and time of the analysis, the excitation beam (including both the wavelength and its associated colour representation), the corresponding laser power, and the list of cameras involved in the measurement. For each camera, the report presents the key beam characterisation parameters, namely the beam tilt angle, the focus position, and the beam waist at the focus expressed in terms of FWHM, as defined in Section 4.1.

In addition to these numerical values, the report includes the FWHM profile as a function of column position, together with the associated hyperbolic fit used to determine the focus point, as well as a beam image with overlays indicating both the beam tilt and the estimated focus position. When characterisation is performed simultaneously for both cameras, the first page of the report provides a summary table of the computed parameters, as illustrated in Figure F.1a. An example of such a report for two cameras is presented in Appendix F.

In parallel with the PDF report, a structured `.csv` file is also generated, containing both the global summary parameters and the complete set of intermediate values used throughout the analysis. The dataset includes fields such as timestamp, camera identifier, excitation beam, power, beam tilt angle, focus position (in both column and row coordinates), the FWHM at the focus in both pixels and micrometers, as well as the individual column positions, peak positions, and corresponding FWHM values along the beam. This comprehensive data structure ensures full traceability of the fitting procedure and enables post-processing, validation, and comparison across different measurements. An example of such a dataset is provided in Appendix G.2.

The images presented in the report are also saved in `.png` format. Among these is the per-column FWHM profile plot, which underpins the calculation of the main beam-characterisation parameters (an example is shown in Figure 4.5). This plot represents the evolution of the FWHM along the analysed beam direction, with each point corresponding to a sampled column in the image. A hyperbolic model is fitted to these values, from which the focus position is estimated as the minimum of the curve, while the corresponding FWHM value defines the beam waist at focus.

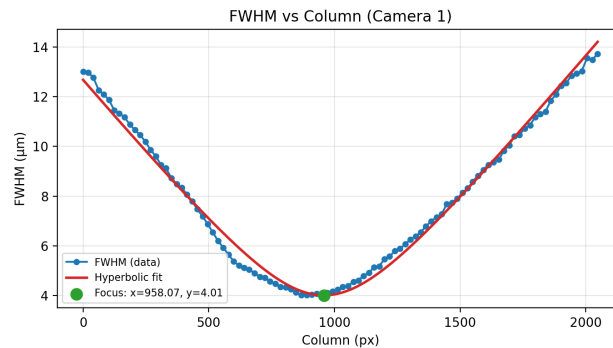


FIGURE 4.5: Per-column FWHM profile (in μm) for Camera 1 associated with the study presented in Appendix F.

Taken together, these outputs integrate numerical parameters, graphical representations,

and annotated images, thereby providing a comprehensive and reproducible characterisation of the beam properties, suitable for routine system alignment and performance verification.

4.3. Beam Characterisation

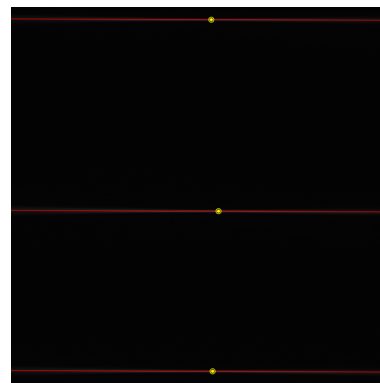
Beam characterisation was carried out using images of the beams at different vertical positions within the camera FOV, corresponding to the top, middle, and bottom regions. These measurements were used to evaluate the LS and its spatial evolution by means of the QC mode of the application (the workflow is described in Section 4.1). All results obtained were compiled in an Excel spreadsheet (Appendix G.3), containing the beam parameters for each camera, solution, excitation beam, and spatial region.

For the different excitation lasers, homogeneous fluorescent solutions were used as the beam-visualisation medium. For the 405 nm laser, Schweppes was used owing to the presence of quinine, which exhibits fluorescence in this spectral region. For 488 nm, fluorescein was used; for 561 nm, rhodamine; and for 640 nm, Nile Blue. These fluorophores provide strong fluorescence emission compatible with their respective excitation wavelengths.

For each excitation condition, measurements were acquired with the corresponding emission filters (Appendix B). The filters used correspond to the standard fluorescence imaging channels, namely DAPI (452/45) for 405 nm, GFP (525/50) for 488 nm, Alexa 568 (610/75) for 561 nm, and Alexa 647 (700/75) for 640 nm. All measurements were performed using the same excitation power of 1 mW, ensuring that the observed differences predominantly reflect wavelength-dependent optical behaviour and alignment effects, rather than variations in illumination intensity.



(A) Beam image at 405 nm (Schweppes).



(B) Beam image at 488 nm (Fluorescein).

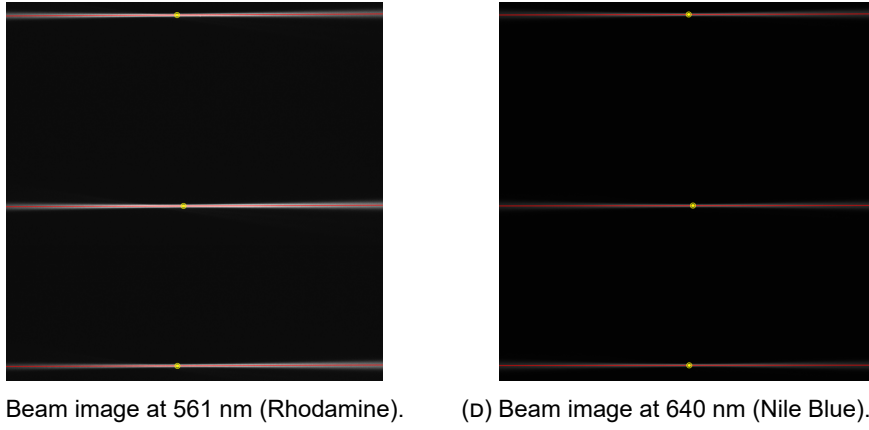


FIGURE 4.6: Beam images in top/middle/bottom of the FOV acquired with Camera 2 for different excitation wavelengths using the corresponding emission filters. The red line indicates the fitted beam tilt, while the yellow markers indicate the detected focus positions at different regions of the image.

The images in Figure 4.6 illustrate the study procedure used for Camera 2 with emission filters, showing the beam positioned in the bottom, middle, and top regions of the FOV, where the red line indicates the fitted beam tilt and the yellow markers denote the detected focus positions at different regions of the image. For both cameras, the same scanner positions corresponding to the top, middle, and bottom of the FOV were used in order to ensure a more reliable comparison.

The measurements reported in Appendix G.2 show that the two detection paths are not perfectly aligned with one another. This is reflected in systematic differences in the measured beam tilt between Camera 1 and Camera 2 under identical conditions. An example for the 488 nm excitation beam, acquired using fluorescein as the fluorescent solution, is shown in Figure 4.7. In this case, Camera 1 presents a beam tilt of 0.626° , whereas Camera 2 presents a beam tilt of 0.233° . The corresponding focus positions also differ, with the focus detected at 958 px for Camera 1 and 1125 px for Camera 2.

The total angular offset between the two detection paths is approximately 0.4° . In practice, this discrepancy was already sufficient to produce measurable differences in the beam parameters recorded by each camera, including the measured tilt and focus position. For this reason, in the present system, an inter-camera angular discrepancy approaching 0.4° was considered a practical upper bound for acceptable relative alignment. Good alignment should therefore correspond to a smaller discrepancy between the two detection paths, while the residual beam tilt measured in each camera should be kept as close as possible to 0° . Owing to this discrepancy, a mechanical adjustment of the camera mount was required after these measurements.

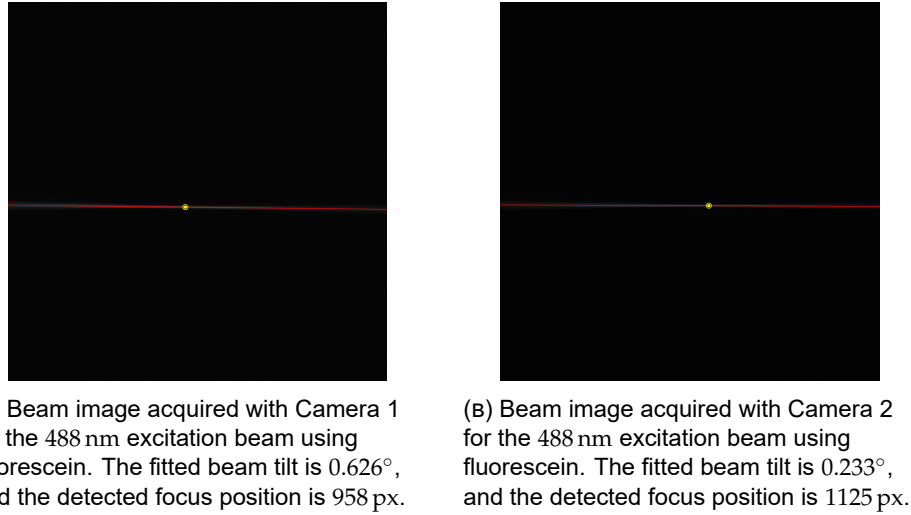


FIGURE 4.7: Comparison between the beam detected by Camera 1 and Camera 2 for the 488 nm excitation beam using fluorescein. The red line represents the fitted beam tilt, and the yellow marker indicates the estimated focus position. The difference in beam tilt and focus position indicates a residual misalignment between the two detection paths.

Furthermore, the beam angle measured in each camera is not equal to 0° . This indicates that the LS is not perfectly parallel to the image plane and therefore exhibits a residual angular tilt across the FOV. This behaviour is evident in Figure 4.6, where the fitted tilt line deviates from a perfectly horizontal orientation. Therefore, the angular mismatch between cameras and the non-zero beam tilt within each camera should be considered as related but distinct alignment limitations: the first reflects a relative misalignment between detection paths, whereas the second reflects the residual inclination of the light sheet itself.

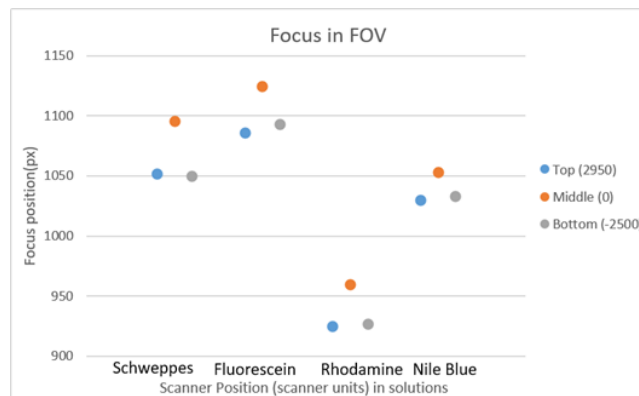


FIGURE 4.8: Focus position measured across the FOV for the different fluorescent solutions and excitation wavelengths. The blue, orange, and grey points correspond to the top, middle, and bottom scanner positions, respectively. The different focus positions between solutions indicate that the excitation beams do not reach focus at the same image coordinate, while the variation between top, middle, and bottom positions shows that the focus also changes slightly across the FOV.

Another relevant observation is that the focus positions of the different beams do not coincide, as shown in Figure 4.8. That is, beams of different wavelengths are not focused at exactly the same location. This suggests the presence of residual wavelength-dependent optical shifts, consistent with a certain degree of chromatic aberration, despite the use of achromatic tube lenses throughout the optical path. In practical terms, this means that the beams are not perfectly co-focused at the same spatial position. This will translate into different lateral focus positions for the samples from channel to channel, impacting the overall image quality.

In addition, the focus position is not constant across the FOV for each wavelength, but instead varies slightly between different regions of the image (Figure 4.6 and 4.8). This indicates that the focus does not remain fixed throughout the FOV. This behaviour is consistent with the fact that the beam tilt is neither constant nor equal to 0° , and therefore the beam does not propagate horizontally across the image.

Another important observation is that the FWHM is not constant across the FOV, indicating that the beam thickness varies throughout the image. This variation reflects both the natural propagation of a Gaussian beam away from its waist and the residual beam tilt discussed previously (Figure 4.8).

These effects have direct implications for both acquisition modes implemented in the software. In confocal mode with rolling shutter, variations in beam position and thickness across the FOV, together with the presence of beam tilt, complicate synchronisation between the beam itself and the active band of the detector, since misalignment arises between the rolling-shutter window and the beam across the image. In widefield mode, the same effect leads to spatially non-uniform illumination, with certain regions of the sample receiving more excitation than others.

It may therefore be concluded that the angular discrepancy between the cameras, the non-zero beam tilt, the wavelength-dependent focus shift, and the spatial variation of the FWHM across the FOV demonstrate that beam alignment in the LS microscope should be regarded as an optical–mechanical problem. These results justify the need for a dedicated beam characterisation procedure and further motivate the implementation of a QC mode in the software as a routine tool for microscope optimisation.

4.4. Field Uniformity

Field uniformity is an important QC parameter in LS microscopy, as image quality depends not only on beam thickness but also on the homogeneity of the excitation distribution throughout the usable FOV. A non-uniform LS results in spatially variable excitation conditions, which can affect fluorescence intensity, image contrast, and the interpretation of image content across the field.

To evaluate field uniformity, one image was acquired for each excitation laser in both confocal and widefield modes, using the corresponding homogeneous fluorescent solutions and associated emission filters. The resulting images were analysed in Fiji [76] with the MetroloJ_QC plugin [79], using brightness and contrast settings spanning the full 16-bit intensity range. This procedure yielded a quantitative estimate of the illumination field uniformity for each beam condition.

The field uniformity values obtained using the plugin for the confocal and widefield configurations are summarised in Tables 4.1 and 4.2, respectively. Camera 2 exhibited the lower residual beam tilt in the beam-characterisation study and was therefore considered more suitable for this analysis. In both modes, field uniformity was highest for the 488 nm beam, whereas the lowest values were observed for the 640 nm beam. This indicates that the light sheet's homogeneity along its propagation axis, and consequently its thickness and intensity, is stable for the 405 nm, 488 nm, and 561 nm laser lines, but not for the 640 nm.

TABLE 4.1: Field-uniformity values obtained in Confocal Mode using Camera 2.

Laser	Solution	Field Uniformity
405 nm	Schweppes (Quinine)	97.2 %
488 nm	Fluorescein	97.6 %
561 nm	Rhodamine	94.7 %
640 nm	Nile Blue	89.1 %

TABLE 4.2: Field-uniformity values obtained in Widefield Mode using Camera 2.

Laser	Solution	Field Uniformity
405 nm	Schweppes (Quinine)	98.0 %
488 nm	Fluorescein	99.4 %
561 nm	Rhodamine	95.0 %
640 nm	Nile Blue	84.8 %

For the 405 nm, 488 nm, and 561 nm excitation conditions, both acquisition modes exhibited field uniformity values above 95%. This indicates that, under these conditions, the LS has an even illumination and detection across the FOV.

Under the 640 nm excitation condition, the field uniformity value was 89.1% in confocal mode and 84.8% in widefield mode. This indicates that, under the bad alignment conditions, confocal mode provides a considerable improvement in field uniformity. This is consistent with the fact that rolling-shutter operation reduces diffuse background signal and restricts detection to the region associated with the LS. For the remaining excitation wavelengths, both modes exhibit similarly high values, with only small differences between them.

An example of this analysis using MetroloJ_QC [79] is shown in Figure 4.9, which presents the field-uniformity image reported by the plugin. This image corresponds to the confocal Nile Blue dataset, for which the measured field uniformity was 89.1%.

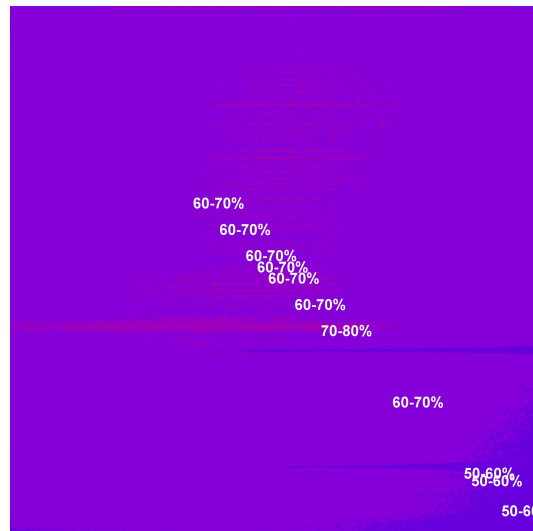


FIGURE 4.9: Example of a MetroloJ_QC [79] field-illumination analysis for the Nile Blue confocal dataset. The report indicates a field uniformity of 89.1%, no saturated pixels, and centering accuracy computed using the 90–100% reference zone. The percentage bands of 60-70%, 70-80%, and 50-60% show an asymmetric, diagonal gradient, with field uniformity falling only within the 50-80% range across much of the FOV. An intensity drop of 20-50% relative to the peak is visible in several areas.

Overall, the field-uniformity analysis indicates that the light sheet microscope delivers highly homogeneous illumination for the 405 nm, 488 nm, and 561 nm excitation conditions, whereas the 640 nm beam remains the most challenging in terms of spatial homogeneity. This result is relevant because previous LS microscopy studies have shown

that field uniformity, field flatness, and channel-dependent focus behaviour are critical for maintaining consistent image quality across large fields of view [80].

This might be caused by issues in the light sheet's propagation axis, or by an uneven beam profile across the FOV. However, given that the tilt at the centre of the FOV is only -0.071° , one possible explanation for this result could be a synchronisation issue between the scanning beam and the rolling shutter's row-by-row readout, which can lead to brightness variations from row to row, as indicated by lines of differing intensity across the image. However, the most likely cause is laser intensity instability, since these lines are also visible, and even more prominent, when images are acquired in widefield Mode (Appendix H).

4.5. Beads

Fluorescent bead samples are an important QC tool, as they provide spatially localised reference emitters that allow the imaging performance of the system to be evaluated directly within the sample volume. Unlike homogeneous fluorescent solutions, which are primarily useful for analysing beam shape and field uniformity, bead samples provide discrete structures whose apparent position, shape, and relative overlap can be inspected directly. This makes them particularly suitable for assessing the spatial correspondence between excitation beams and for identifying misalignment effects at the sample plane.

In this work, multicolour bright beads with a nominal diameter of $3\text{ }\mu\text{m}$ and multicolour dim beads with a nominal diameter of $2\text{ }\mu\text{m}$, embedded in agarose, were employed for QC. These multicolour beads can be excited and will emit fluorescence in the four ranges: 405 nm, 488 nm, 561 nm, and 640 nm. The resulting multicolour bead dataset enabled the chromatic shift assessment between the different excitation conditions directly within the same sample volume.

Figure 4.10 shows a three-dimensional image of the beads used in this study, visualised with Imaris Viewer [81]. In this representation, yellow corresponds to the 405 nm beam, cyan to 488 nm, red to 561 nm, and green to 640 nm. It can be seen that the beads are not perfectly superimposed. Instead, a relative displacement between the channels is observed along the Y axis, that is, along the acquisition planes. In practical terms, the beams appear to diverge from one another along Y , which is consistent with a displacement in the excitation geometry. This might be a result of the LS itself not being perfectly co-planar across the different wavelengths due to chromatic aberrations introduced by the

illumination optics, or because the different laser lines might not be perfectly aligned in relation to one another before entering the illumination objective. Another possible explanation for the apparent Y -offset, even though the bead itself didn't move, might be related to the detection objective and its optics, which can introduce wavelength-dependent focus shifts. However, since the system uses plan fluor objectives, and the tube lenses are also corrected between 350-700 nm, the probable cause for the Y -offset observed is microscope misalignment.

At the same time, a white region can also be observed in the image shown in Figure 4.10, indicating the overlap between the 405 nm (yellow) and 488 nm (cyan) signals. This suggests partial spatial coincidence between these two channels.

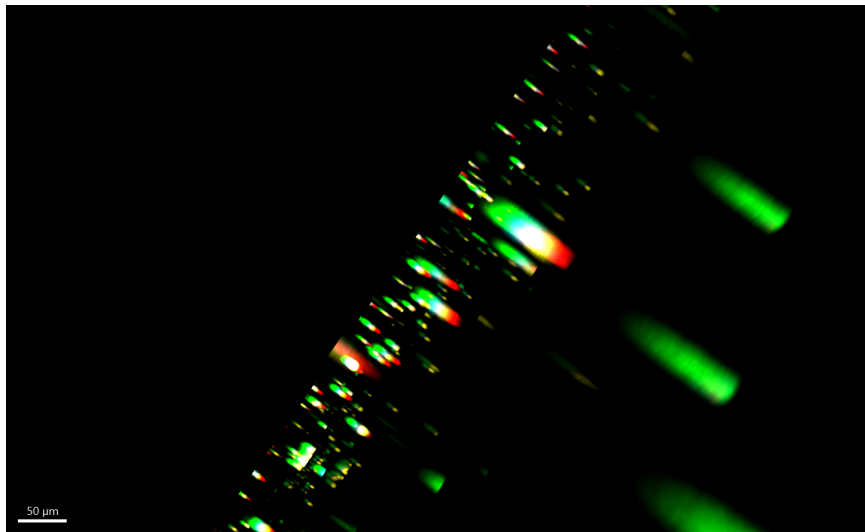


FIGURE 4.10: Three-dimensional image of fluorescent beads embedded in agarose, used to assess the spatial correspondence between excitation channels. The colour assignment is 405 nm in yellow, 488 nm in cyan, 561 nm in red, and 640 nm in green. The relative displacement between coloured bead signals suggests a residual spatial misalignment between beams at the sample plane, particularly along the Y direction, although local white regions indicate partial overlap between the 405 nm and 488 nm signals.

Overall, the bead analysis indicates that multicolour excitation in the LS microscope is still affected by a small but measurable spatial offset, particularly along the Y axis. This finding reinforces the importance of bead-based verification as part of the QC workflow, since such micrometric shifts may not be noticeable by eye yet can still affect the spatial correspondence between channels in biological imaging.

5. Microscope Debugging

The LS microscope software integrates multiple hardware devices, real-time camera visualisation, scanner control, acquisition routines, QC tools, and user interface interactions. In such a complex system, errors and failures may arise from multiple sources at both the hardware and software levels. For this reason, a dedicated logging system was implemented to support debugging, operational traceability, and the long-term maintenance of the microscope.

The purpose of the logging system is to provide a continuous chronological record of hardware and software events throughout microscope operation, which facilitates distinction between hardware- and software-related issues, and supports routine debugging during microscope operation.

5.1. Log File Implementation

A log file was implemented in the LS microscope software to record operational events and error conditions during use. The logging system was implemented through a central logging module, which is initialised during software start-up. Once the application is launched, the logging module is created and reused across different parts of the program, allowing events generated by the cameras, lasers, filter wheels, scanner, acquisition routines, quality-control tools, and software mode transitions to be recorded in a common chronological file.

At program start-up, the user selects the working directory in which the generated data are to be stored. Following this selection, a `logs` folder is created within the selected directory, or reused if it already exists, and a session-specific `.txt` log file is stored in this folder. This organisation ensures that the operational history of the microscope remains associated with the acquisition and quality-control data generated during the same session. Consequently, if an error occurs, the log file can be inspected alongside the corresponding output data, thereby improving traceability and simplifying subsequent debugging.

The log file uses a structured format as follows:

```
(date) (time) [(log level)] (name) | (thread name) | (message)
```

This format makes it possible to identify what occurred, when it occurred, and in which execution context it occurred. The inclusion of the thread name is particularly useful for distinguishing between events generated by the GUI and those generated by background processes, allowing the source of errors to be identified more precisely and corrected accordingly. The logging file is also configured with a dynamic saving over time – preventing uncontrolled growth during long sessions and preserving previous log files as backups.

At the code level, the logging system follows an asynchronous architecture. Rather than writing each log entry directly to the file from the thread that generated the event, log records are first placed in a queue and subsequently written to the logging file by a background process. This design is particularly important in a microscope-control application, since synchronous file writing could block the graphical interface or delay routines associated with acquisition and QC, thereby reducing the responsiveness of these processes.

Consequently, events generated by the hardware, acquisition routines, and quality-control procedures can be recorded rapidly without requiring the originating thread to wait for the operating system to complete a file-writing operation. This reduces the probability of GUI freezing, visualisation delays, loss of acquisition synchronisation, and reduced responsiveness during high-frequency operations.

Asynchronous logging also provides increased robustness in an application composed of multiple widgets connected to different hardware devices and operating in a multithreaded environment. This is because different modules may generate log events simultaneously during microscope operation. The use of a queue therefore provides a controlled mechanism for collecting messages from the different active widgets, before writing them sequentially to the log file. This preserves a consistent logging output and avoids simultaneous file access by multiple threads. This approach is particularly useful in the LS software, where camera acquisition, device communication, scanner control, acquisition routines, QC calculations, and graphical interaction are handled by separate widgets and worker threads.

The logger was designed to record a broad range of operational events. These include camera live-view activation and deactivation, camera snapshots, camera restart operations, laser on/off state changes, laser intensity changes, camera-mode transitions, scanner operation in Widefield or Confocal Mode, hardware connection events, transitions between Acquisition and Quality Control Mode, acquisition-related events, filter changes,

and events occurring during QC analysis. Recording these events in the same chronological file provides a detailed history of the microscope state before, during, and after each operation.

Figure 5.1 illustrates the complete logging workflow implemented in the light sheet microscope software. After the software starts, the user selects the working directory, where in a dedicated `logs` folder, a corresponding `.txt` log file are created. The logger is then initialised, the initial hardware connections are recorded, and the software enters its normal running state. During operation, each hardware event, software event, or runtime error is sent to the logging queue and written asynchronously to the log file, without interrupting execution. This process continues until the microscope software is closed, after which the log file can be used for debugging and traceability.

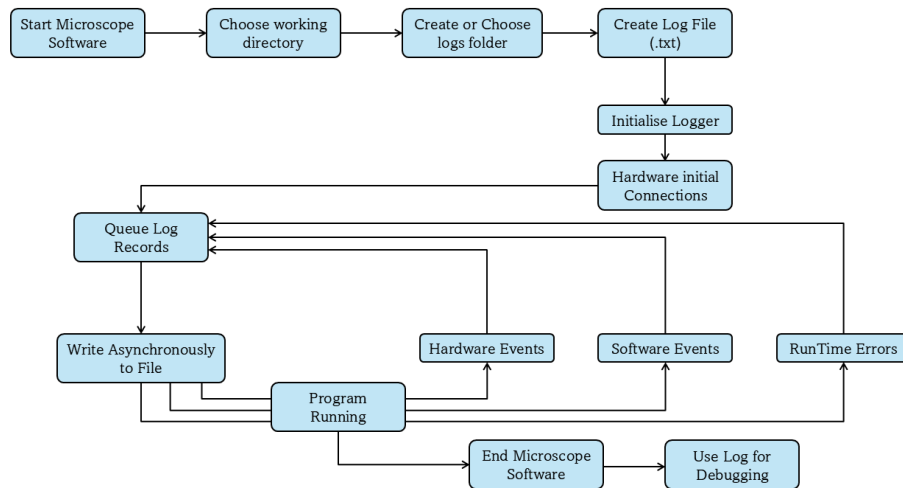


FIGURE 5.1: Schematic representation of the logging workflow implemented in the ALM microscope software. After the user selects the working directory, in a dedicated `logs` folder, a `.txt` log file are created. During operation, hardware events, software events, and runtime errors are continuously queued and written asynchronously to the log file while the program remains running. The resulting log can then be used for debugging and traceability after the software session ends.

5.1.1 Hardware Log

The hardware log records events associated with the physical devices of the microscope, including the lasers, cameras, filter wheels, scanner, and stages. During software initialisation, the log file documents whether each device was detected and successfully initialised. Where available, connection details such as communication ports, serial numbers, controller type, and device status are recorded for the corresponding hardware components.

This hardware-level logging is important because the LSFM relies on the coordinated operation of several independent devices. A failure in one device may not prevent the graphical interface from opening, but it can compromise acquisition, real-time visualisation, scanner operation, filter selection, or QC functionality. Consequently, recording the start-up state of each device provides an immediate diagnostic tool for identifying missing, disconnected, or incorrectly configured hardware.

In addition, the device state during program operation is also logged. For the cameras, all events related to enabling or disabling live visualisation in napari are recorded, together with snap acquisition events and camera restart events. These entries are useful for distinguishing between a camera connection failure during start-up and a failure occurring during software execution.

For the lasers, on/off events are recorded for each excitation wavelength, together with changes in laser power expressed in mW. This is particularly useful during acquisition and QC, as it allows the user to verify whether the expected excitation line and intensity were active at the correct time.

For the filter wheels, emission-filter change events are recorded, enabling the optical configuration used during multichannel imaging or beam-characterisation routines to be traced.

For the scanner, the log records whether the system is operating in widefield or confocal mode, which is closely linked to the selected camera mode. This allows the scanner behaviour at a given moment to be characterised, since its operation differs between the two modes, as described in Chapter 3.

Overall, the hardware log provides a structured record of the physical state of the microscope. It is useful both during software development and routine microscope operation, as it enables the user to verify which devices were connected, how they were configured, and whether any hardware component exhibited failures before or during an experiment.

5.1.2 Software Log

The software log records events associated with the internal execution of the application. These include software initialisation, creation of the GUI, user-triggered actions, mode transitions, acquisition and QC events, and unexpected runtime errors. Since each log entry also contains the thread name, the log file provides information about the execution

context in which each event occurred. This is particularly useful in the software, where multiple operations are executed through independent widgets and worker threads.

One of the main events recorded at the software level is the transition between Acquisition Mode and Quality Control Mode. Since each mode exposes a different widget in the GUI, recording these transitions enables the operational state of the software to be reconstructed at the time of a given event or failure. For example, an error occurring during QC analysis can be distinguished from one occurring during image acquisition, live visualisation, or camera-mode switching.

All events associated with device-specific widgets are recorded. The interaction between software and hardware provides important diagnostic information, enabling a distinction to be made between hardware communication problems and errors caused by software-side operations, such as an invalid camera state or a failed live-visualisation request.

In addition, acquisition-related events are recorded. These include the start of an acquisition sequence, acquisition progress, hardware state changes triggered during acquisition, and operations associated with file saving. Since a Y-stack acquisition relies on the coordinated behaviour of the cameras, lasers, scanner, stages, and filter wheels, the software log provides a chronological record that helps determine whether a problem occurred before acquisition, during hardware configuration, during image capture, or during file writing.

Events related to QC are also recorded. The QC workflow includes real-time calculations, saving of calculated results, background execution of numerical analyses, overlay generation, FWHM plotting, and report/data export. Recording these events is important because QC depends on real-time camera frames, numerical fitting routines, graphical updates, and file-generation operations. If an error occurs, the log file helps determine whether the failure originated from unavailable camera frames, invalid user-defined parameters, failure of the numerical analysis, overlay generation, or report-export operations.

In addition to explicit software events, the logging system also captures Qt messages and unhandled Python exceptions through handlers installed during application start-up. This allows graphical-interface warnings and unexpected runtime exceptions to be recorded in the same file as normal operational events. As a result, the log file provides a unified record of expected actions and unexpected failures, which is essential for debugging a

complex graphical microscope-control application.

The software log is therefore complementary to the hardware log. Whereas the hardware log records the state of the physical devices, the software log records how those devices are used by the application. Together, both types of log are stored in a single file and allow the complete execution sequence of a microscope session to be reconstructed, from software and hardware initialisation through acquisition and QC to shutdown. This improves traceability and provides an effective basis for identifying, reproducing, and correcting errors in the ALM microscope software.

5.2. Types of errors reported

The implemented logging system records errors associated with both hardware and software, as described in Sections 5.1.1 and 5.1.2, respectively. Hardware-related errors include missing devices during software initialisation, communication failures with instruments connected via serial or VISA interfaces, incomplete device initialisation, and loss of device availability during execution. Software-related errors include widget initialisation failures, invalid device states, failed activation of live visualisation, runtime exceptions, and warnings generated by the graphical interface.

The log file classifies events into three levels: `INFO`, `WARNING`, and `ERROR`. Informative messages are used to confirm successful operations, such as device connections, changes in laser state, or activation of real-time visualisation. Warning messages report non-critical issues that do not necessarily prevent the program from continuing execution. Error messages indicate failures that prevent an operation from being completed as expected. This classification enables the user to rapidly identify the severity and origin of a given event.

Figure 5.2 shows two representative examples of errors captured by the logging system. In the first example (Figure 5.2a), the log file reports a hardware connection problem during software start-up. Since the laser box was switched off, no valid laser-controller port was detected. As a result, the laser box could not be connected to the corresponding software widget. This information is particularly useful because it identifies not only the device that failed, but also the software location and communication layer associated with the failure.

In the second example (Figure 5.2b), the software started correctly and all devices were successfully connected. The log records normal operational events, including successful camera connection, activation of live visualisation, and changes in laser state. Camera 1

was then deliberately disconnected during execution. The log subsequently records the error associated with the failed attempt to start live visualisation from that camera. This demonstrates that the logging system is able to detect not only start-up failures, but also runtime failures that occur after the microscope software has already entered its normal operational state.

```

2026-06-25 13:12:14 [INFO] ALM | MainThread | Log directory updated to: logs
2026-06-25 13:12:14 [INFO] ALM | MainThread | Main window created successfully
2026-06-25 13:12:14 [INFO] ALM | MainThread | Entering Qt event loop
2026-06-25 13:12:14 [INFO] ALM | MainThread | ALM Lightsheet initialisation started
2026-06-25 13:12:14 [INFO] ALM | MainThread | ALM Lightsheet initialisation completed successfully
2026-06-25 13:12:14 [ERROR] ALM | LASERS | Laser box not found. No 'Coherent OBIS Device' port was detected.
2026-06-25 13:12:14 [ERROR] ALM | FILTER WHEEL | Filter wheel 2 connection attempt failed. Port=COM4, serial=AD03DTC7A
Traceback (most recent call last):
  File "C:\Users\ALM_Light_Sheet\Desktop\VitaSlice - Acquisition Mode YLambda\Extra_Files\Devices_Connections.py", line 87, in filterwheel_2
    filter_conn_2 = _ZaberConnection(port=p.device, baudrate=115200, timeout=0.05)
    ~~~~~^~~~~~
  File "C:\Users\ALM_Light_Sheet\anaconda3\envs\env_napari7\Lib\site-packages\microscope\controllers\zaber.py", line 123, in __init__
    self._serial = serial.Serial(
    ~~~~~^~~~~~
  File "C:\Users\ALM_Light_Sheet\anaconda3\envs\env_napari7\Lib\site-packages\serial\serialwin32.py", line 33, in __init__
    super().__init__(*args, **kwargs)
  File "C:\Users\ALM_Light_Sheet\anaconda3\envs\env_napari7\Lib\site-packages\serial\serialutil.py", line 244, in __init__
    self.open()
  File "C:\Users\ALM_Light_Sheet\anaconda3\envs\env_napari7\Lib\site-packages\serial\serialwin32.py", line 64, in open
    raise SerialException("could not open port {}: {}".format(self.portstr, ctypes.WinError()))
serial.SerialException: could not open port 'COM4': PermissionError(13, 'Access is denied', None, 5)
2026-06-25 13:12:15 [INFO] ALM | FILTER WHEEL | Filter wheel 1 connected successfully. Port=COM9, serial=A10K5RCPA
2026-06-25 13:12:15 [INFO] ALM | SCANNER | Scanner connected successfully. DLL=RTCSOLLx64, init_result=0
2026-06-25 13:12:15 [INFO] ALM | FILTER WHEEL | Filter wheel 2 connected successfully. Port=COM4, serial=AD03DTC7A
2026-06-25 13:12:18 [INFO] ALM | STAGES | Stages connected successfully. Controller=C-884, serial=118067518
2026-06-25 13:12:21 [INFO] ALM | CAMERA | Camera 1 connected successfully
2026-06-25 13:12:22 [INFO] ALM | CAMERA | Camera 2 connected successfully
2026-06-25 13:12:22 [INFO] ALM | SCANNER | Scanner initialised successfully. Device type: WinDLL
2026-06-25 13:12:23 [ERROR] ALM | LASERS | Laser controller was not provided to Lasers_Widget.
2026-06-25 13:12:23 [ERROR] ALM | LASERS | Failed to initialise the laser controller in Lasers_Widget.

```

(A) Example of hardware connection failures recorded during software start-up. The laser box was switched off, resulting in the message that no valid Coherent OBIS device port was detected.

```

2026-06-25 13:10:00 [INFO] ALM | FILTER WHEEL | Filter wheel 1 connected successfully. Port=COM9, serial=A10K5RCPA
2026-06-25 13:10:00 [INFO] ALM | SCANNER | Scanner connected successfully. DLL=RTCSOLLx64, init_result=0
2026-06-25 13:10:00 [INFO] ALM | LASERS | Laser box connected successfully. Port=COM7, VISA resource=ASRL7::INSTR
2026-06-25 13:10:01 [INFO] ALM | FILTER WHEEL | Filter wheel 2 connected successfully. Port=COM4, serial=AD03DTC7A
2026-06-25 13:10:04 [INFO] ALM | STAGES | Stages connected successfully. Controller=C-884, serial=118067518
2026-06-25 13:10:08 [INFO] ALM | CAMERA | Camera 1 connected successfully
2026-06-25 13:10:09 [INFO] ALM | CAMERA | Camera 2 connected successfully
2026-06-25 13:10:09 [INFO] ALM | SCANNER | Scanner initialised successfully. Device type: WinDLL
2026-06-25 13:10:09 [INFO] ALM | LASERS | Laser controller attached to Lasers_Widget. Device type: SerialInstrument
2026-06-25 13:10:09 [INFO] ALM | LASERS | Laser controller initialised successfully: channels 2-5 set to AM OFF and Internal GSP mode.
2026-06-25 13:10:09 [INFO] ALM | MainThread | UI mode transition: None -> acquisition
2026-06-25 13:10:13 [WARNING] ALM | MainThread | QObject::connect(QStyleHints, QStyleHints): unique connections require a pointer to member function of a QObject subclass
2026-06-25 13:10:23 [INFO] ALM | CAMERA | Camera 1 live view started
2026-06-25 13:10:24 [INFO] ALM | LASERS | Laser state changed: Laser=488 nm, channel=4, state=ON, power=1.0 mW
2026-06-25 13:10:25 [INFO] ALM | LASERS | Laser state changed: Laser=488 nm, channel=4, state=ON, power=2.0 mW
2026-06-25 13:10:26 [INFO] ALM | LASERS | Laser state changed: Laser=488 nm, channel=4, state=ON, power=1.0 mW
2026-06-25 13:10:27 [INFO] ALM | LASERS | Laser state changed: Laser=488 nm, channel=4, state=OFF, power=0 mW
2026-06-25 13:10:28 [INFO] ALM | CAMERA | Camera 1 live view stopped
2026-06-25 13:10:46 [ERROR] ALM | CAMERA | Failed to start Camera 1 live view

```

(B) Example of a runtime camera failure recorded after successful software initialisation. The camera was deliberately disconnected after the program had started in order to verify that the logging system correctly recorded a live-view failure.

FIGURE 5.2: Representative hardware and runtime faults captured by the ALM microscope logging system.

These examples highlight the diagnostic value of storing both successful operations and failures within the same chronological file. Successful connection messages provide a reference state for the system, whereas subsequent warning or error messages indicate when and where a failure occurred. This makes it possible to distinguish between a device that was never correctly initialised and a device that failed during operation.

Overall, the logging system provides an effective debugging mechanism for the microscope software. By recording hardware events, software actions, runtime errors, and exception stack traces in a structured chronological format, it supports rapid diagnosis of

system failures and improves the robustness and maintainability of the microscope-control environment.

6. Conclusion & Future Work

6.1. Discussion & General Conclusions

This dissertation contributes to the development, validation, and characterisation of the custom control software for the ALM LSM. The main objective was to expand the existing microscope controlling software by implementing new functionalities for acquisition, quality control, and debugging, as well as to evaluate their practical impact on microscope operation and image quality.

A major contribution of this work was the implementation of a confocal acquisition mode based on rolling-shutter synchronisation between the camera and the scanner. Unlike widefield mode, in which fluorescence is integrated over the entire detector, confocal mode synchronises the active detector band with the instantaneous position of the scanned LS. This enables improved rejection of diffuse and out-of-focus light contributions and enhances the visual separation between different fluorescence channels within an image. The comparison between widefield and confocal acquisition showed that the confocal mode can improve image contrast and structural definition, particularly in samples with significant out of focus light, such as spheroids and hydrogels.

In addition to implementing the confocal mode, improvements were introduced to the system control logic. In widefield mode, laser emission was restricted to the effective scanning phase, thereby avoiding unwanted illumination during the scanner return motion. This correction eliminated an artefact that appeared as a high-intensity band in the image, highlighting the importance of explicit laser control even outside rolling-shutter-based acquisition.

A second contribution was the development of a Quality Control mode within the microscope software. In this mode, the user can characterise the generated light sheet through the same GUI used for acquisition. The QC workflow performs Gaussian fitting of the beam profiles, estimates the beam tilt, calculates the focus position, and determines the FWHM at the focus. The integration of numerical results, napari overlays, and FWHM plots provides immediate feedback to the user and enables real-time microscope alignment.

In addition, the characterisation study can be saved in report format. The software generates an output folder containing a PDF report, structured `.csv` data, annotated beam images, and FWHM plots. This provides reproducible documentation of the beam-characterisation process and allows the extracted data to be reused for subsequent validation or comparison between measurements. The availability of both visual and quantitative outputs makes the QC mode suitable for routine system verification and alignment.

For the characterisation of the beam in the ALM microscope, the software QC mode was used, revealing that alignment remained a critical factor affecting system performance. The two detection paths exhibited an angular discrepancy of approximately 0.4° between the two cameras, which was sufficient to degrade acquisition quality. In addition, the beam tilt was not equal to 0° across the image, indicating that the light sheet was not perfectly parallel to the image plane. Finally, the focus position was not constant, and the FWHM varied across the beam, showing that the beam width was not uniform throughout the image.

Taken together, these observations indicate that the beam was not perfectly aligned and that the two cameras were not fully aligned with respect to one another. These effects have direct consequences for both acquisition modes: in confocal mode, they impact in the synchronisation between the rolling-shutter window and the beam; in widefield mode, they contribute to non-uniform illumination of the sample.

In addition to beam characterisation, a complete QC assessment was done by looking at the field-uniformity and chromatic co-registration. The system exhibited high field-uniformity values for the 405 nm, 488 nm, and 561 nm excitation conditions, whereas the 640 nm condition showed lower homogeneity. These results highlight that beam confinement, angular alignment, and illumination uniformity are strongly interconnected aspects of microscope performance.

Multi-color fluorescence bead analysis provided additional sample-level validation of the system alignment. A residual spatial displacement between the excitation channels, along the Y axis was found upon analysis of the beads images. This Y -offset was likely on the order of a few micrometres, being clearly visible in the bead datasets even when not obvious through direct visual inspection during beam alignment. This confirms the importance of bead-based quality-control checks for detecting small spatial offsets between channels, which may affect the interpretation of multicolour biological imaging.

Finally, a logging system was also implemented to improve debugging and system traceability. The logger records hardware events, software events, runtime errors, and exception stack traces in a structured chronological format. Logging is performed asynchronously, preventing file-writing operations from blocking the graphical interface or interfering with acquisition processes by making them slower. This is particularly important in a multithreaded microscope-control application, where cameras, scanner control, device communication, acquisition routines, and QC calculations may run in parallel.

In conclusion, the work carried out in this dissertation improved the capabilities of the ALM LSM by implementing a synchronised confocal acquisition mode based on rolling shutter, integrating a beam quality-control system, demonstrating the importance of field-uniformity assessment and bead-based validation for microscope performance, and introducing a robust event-logging framework. These developments contribute to a more reliable, traceable, and user-oriented microscope-control environment. At the same time, the results show that hardware alignment and optical stability remain limiting factors, motivating further improvements in both the mechanical design and the software acquisition workflow.

During the development of this work, Artificial Intelligence (AI) tools were used as complementary support for code development and refinement, language correction and text improvement. These tools were mainly used to improve clarity and grammatical correctness.

6.2. Future Work

Although the developments presented in this dissertation have improved the image-acquisition, quality-control, traceability, and debugging capabilities of the ALM microscope, several aspects can still be improved in future work. These improvements include both hardware-related developments, aimed at increasing optical and mechanical stability, and software-related developments, focused on improving acquisition scalability, data processing, and the user experience.

6.2.1 Hardware

An important hardware improvement would be the development of a new sample chamber with greater mechanical stability. Ideally, the chamber should be fixed to the optical table or to a mechanically rigid reference structure.

A stable chamber would facilitate focusing of both the detection and illumination paths after chamber replacement or objective cleaning, as in the current set up, the chamber is sealed with Picodent that physically connects it to the objectives, so changing the focus position of one objective causes the entire chamber to move which impacts the capacity for dual side detection.

Another relevant improvement would be the implementation of a post-scanner alignment system based on two mirrors arranged in a geometry complementary to that of the scanner. Such a configuration would allow optimisation of the beam incidence and polarisation at the sample. Since the propagation direction and polarisation state of the excitation beam can influence light sheet formation and image quality, this additional alignment stage could contribute to improved LS imaging performance.

Fine adjustment of the upper scanner mirror should also be considered as a future hardware optimisation step. The bead analysis suggested a residual Y-offset between excitation channels at the sample plane, and adjustment of this mirror could help correct small lateral displacements of the scanned beam. This optimisation should be guided by the QC workflow and verified using multicolour beads, allowing beam registration to be assessed quantitatively after each adjustment.

In addition, future hardware optimisation should include a more systematic alignment procedure assisted by the QC mode developed in this work. The QC workflow provides quantitative feedback on beam tilt, focus position, and FWHM, and can therefore be used as an alignment aid rather than only as a diagnostic tool. Future work should define acceptable tolerance ranges for these parameters and use them to guide mechanical and optical adjustments of the system. This would make alignment more reproducible and less dependent on subjective visual inspection.

6.2.2 Software

From the software perspective, an important future improvement would be the integration of stitching directly into the acquisition workflow. This would allow the user to acquire multiple adjacent fields of view and reconstruct larger sample regions without relying on external post-processing tools. Such an implementation would be particularly useful for biological samples that exceed the camera FOV and should include the storage of tile position, % of overlap, acquisition order, stitching method, and associated metadata.

Another important development concerns image saving during acquisition. Future versions of the software should implement frame-by-frame saving using a non-blocking strategy, preventing the GUI from freezing during large acquisitions. A possible solution would be a producer–consumer architecture, in which acquired frames are placed in a queue by the acquisition thread and written to disk by a separate background worker. This approach would be conceptually similar to the asynchronous logging system implemented in this work.

Finally, future software improvements could further expand the QC workflow. The software could compare new QC measurements with reference values and alert the user when parameters such as beam tilt, focus position, FWHM, field uniformity, or bead-based channel alignment exceed predefined tolerance limits. This would make the QC mode not only a diagnostic tool, but also a routine monitoring tool for microscope stability.

Overall, future software development should focus on improving acquisition scalability, non-blocking data handling, and automated quality control. These improvements would make the ALM software more robust, responsive, and suitable for large multidimensional datasets.

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A. Immunofluorescence techniques

The fluorophore can either be conjugated to the primary antibody (direct), which recognizes the antigen, or to a secondary antibody (indirect), which recognizes the primary.

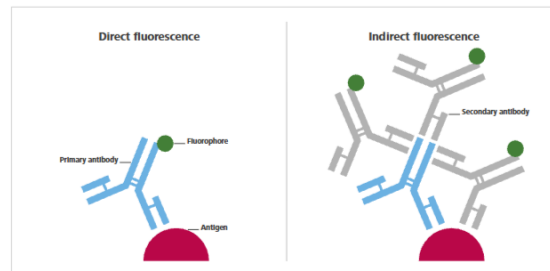


FIGURE A.1: Illustration of immunofluorescence techniques (from Ref. [6]).

Advantages of direct immunofluorescence include a shorter protocol and less chance of cross-reactivity, but the greatest disadvantage is the low signal intensity. With indirect immunofluorescence, multiple secondary antibodies may bind the same primary antibody to amplify the fluorescent signal.

B. Filters available on ALM LSFM

The filters available in each filter wheel, together with their target fluorophores and spectral characteristics, are presented in Tables B.1 and B.2.

TABLE B.1: Filter configuration in Filter Wheel 1.

Wheel Position	Target Fluorophore	Transmission range
0	DAPI	452/45 nm
1	GFP	525/50 nm
2	YFP	545/25 nm
3	Alexa 568	610/75 nm
4	Alexa 647	700/75 nm

TABLE B.2: Filter configuration 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

C. Supplementary Multimedia

C.1. ALM DLSM photographs

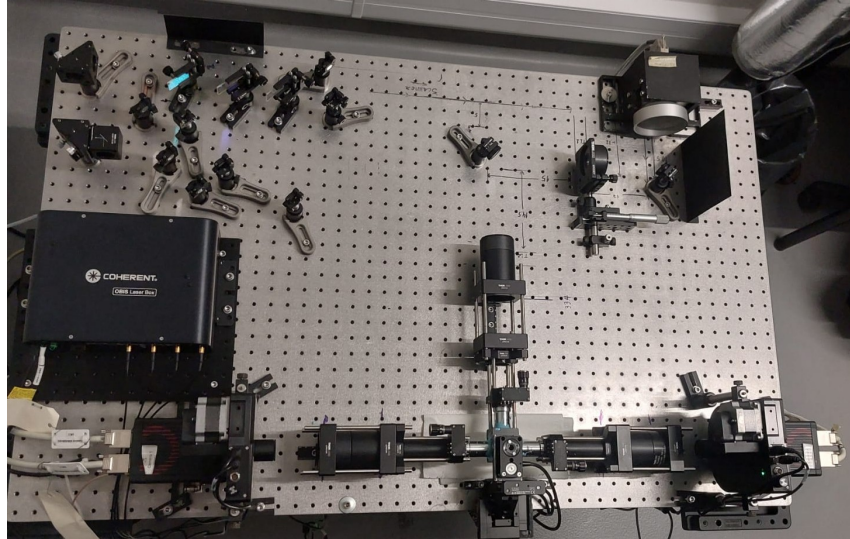


FIGURE C.1: Top view of the ALM light sheet microscope.

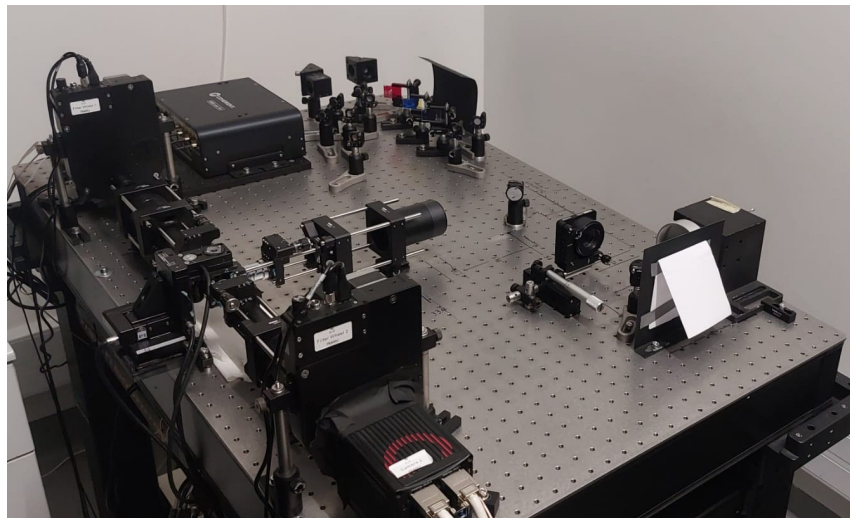


FIGURE C.2: Angled view of the ALM light sheet microscope.

D. Experimental Calibration Data for Synchronisation in Confocal Mode

For calibration of the confocal mode, the minimum scanner speed required for the scanned beam to correctly fill the detector FOV was determined for each selected camera exposure time. The corresponding values are presented in Table D.1.

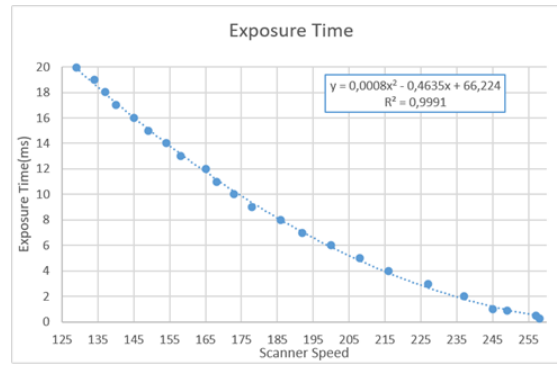
TABLE D.1: Experimental calibration data for confocal synchronisation.

Exposure Time (ms)	Scanner Speed (scanner units)
19.956451	129
19.001504	134
18.007579	137
17.003910	140
16.000241	145
15.006316	149
14.002647	154
13.008722	158
12.005053	165
11.001383	168
10.007459	173
9.003789	178
8.000120	186
7.006195	192
6.002526	200
5.008602	208
4.004932	216
3.001263	227
2.007338	237
1.003669	245
0.906226	249
0.506707	257
0.272842	258

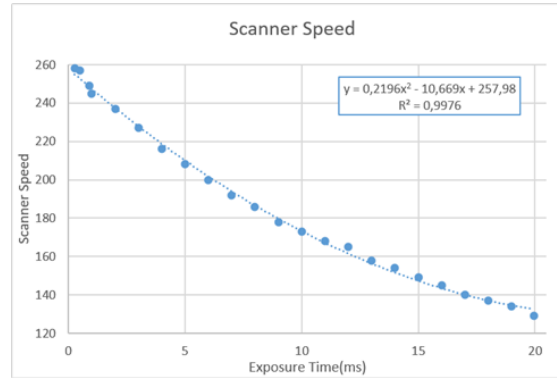
The relationships between exposure time and scanner speed obtained from the experimental calibration are shown in Figure D.1 and are described by Equations D.1 and D.2.

$$t_{\text{exp}} = 0.0008 \cdot v_{\text{scanner}}^2 - 0.4635 \cdot v_{\text{scanner}} + 66.224 \quad (\text{D.1})$$

$$v_{\text{scanner}} = 0.2196 \cdot t_{\text{exp}}^2 - 10.669 \cdot t_{\text{exp}} + 257.98 \quad (\text{D.2})$$



(A) Quadratic fit of scanner speed as a function of exposure time.



(B) Quadratic fit of exposure time as a function of scanner speed.

FIGURE D.1: Experimental calibration curves used to define the synchronisation relationship between (a) camera exposure time and (b) scanner speed in Confocal Mode.

E. Supplementary Comparison between Wide-field and Confocal Mode

This appendix presents a supplementary comparison between Widefield and Confocal acquisition modes using neural crest cell spheroid samples, complementing the comparisons presented in Chapter 3. While the blood vessels labelled zebrafish (Subsection 3.3.1) and hydrogel (Subsection 3.3.2) samples showed clearer differences between the two modes, the neurosphere dataset provides an additional example of the visual differences that can be observed in a biological sample.

All neurosphere images were processed using Fiji [76], with identical brightness and contrast settings applied to each pairwise comparison. The comparison presented here was performed using average-intensity Y projections, which made the differences between Confocal and Widefield acquisition more visible.

E.1. Neural crest cell spheroid Sample

A representative comparison of a neural crest cell spheroid sample acquired in Confocal Mode and Widefield Mode is shown in Figure E.1, where neural crest cells are labelled. In this comparison, only the fluorescence signal associated with the 488 nm excitation laser is shown. In both acquisition modes, the neural crest cell spheroid sample presents an approximately spherical morphology, with fluorescence distributed throughout the internal region of the sample. However, clear visual differences between the two modes can be observed.

In the confocal-mode image (Figure E.1a), the background surrounding the spheroid appears darker, and the sample boundary is more clearly separated from the surrounding signal. Internal fluorescence intensity variations are also more distinguishable, indicating improved local contrast within the spheroid.

In contrast, the widefield-mode image (Figure E.1b) exhibits a broader diffuse signal around the spheroid and a more pronounced high-intensity region within the sample. This increased diffuse contribution partially reduces the visibility of local internal structures and decreases the apparent contrast between the spheroid and the background.

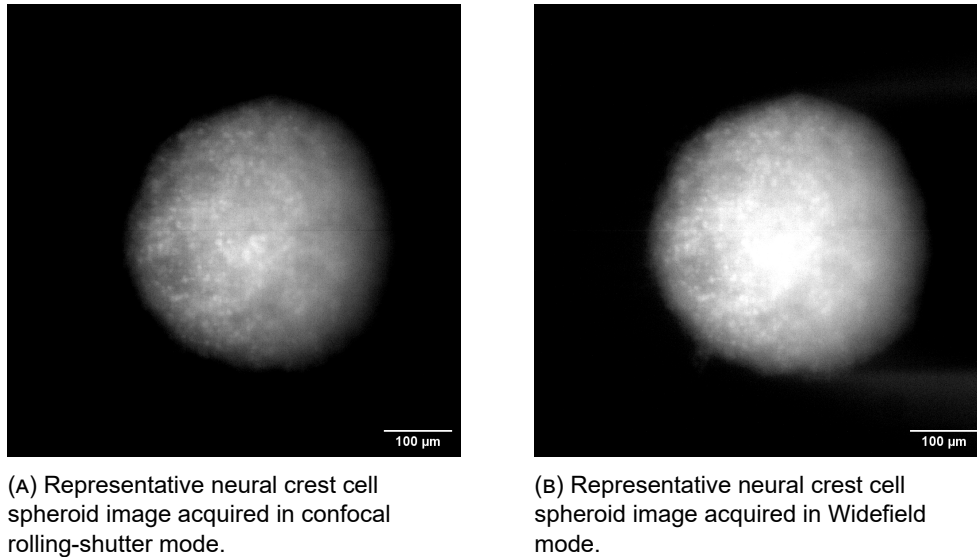


FIGURE E.1: Qualitative comparison between (a) confocal rolling-shutter mode and (b) widefield mode for a neural crest cell spheroid sample imaged with the 488 nm excitation laser. Both images were acquired with Camera 1 at a sample angle rotation of 0° and are displayed using the same brightness and contrast settings. The confocal image exhibits improved local contrast relative to the Widefield image.

These observations are consistent with the expected behaviour of the two modes: in widefield imaging, fluorescence is integrated over the entire FOV of the detector, leading to increased background and out-of-focus contributions. In contrast, the synchronised rolling-shutter detection employed in confocal mode reduces unwanted out-of-focus light signal and improves local contrast.

F. Example of QC Report

This appendix presents an example of a Quality Control report (Chapter 4) for the light-sheet beam corresponding to the blue laser (488 nm), measured using fluorescein at the centre of the FOV with a GFP emission filter. The corresponding .csv data associated with this study are available in Appendix G.2.

Quality Control Report (Both Cameras)

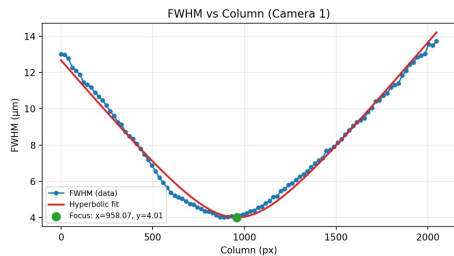
Timestamp: 2026-04-01T11:46:41
Beam used: 488 nm ■
Intensity: 1 mW
Included cameras: Camera 1, Camera 2

Camera	Angle Tilt (deg)	Focus (col,row)	Beam Waist at focus
Camera 1	0.626	(958,1107.83)	4,006 μm
Camera 2	0.233	(1125,1100.09)	3.872 μm

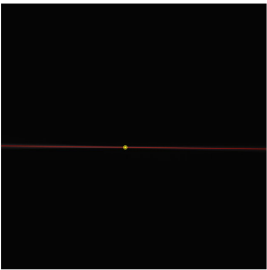
(A) Page 1 of the QC report.

Results - Camera 1

Angle Tilt (deg): 0.626
Focus (col,row): (958,1107.83)
Beam Waist (FWHM at focus): 4.006 μm
FWHM graph:



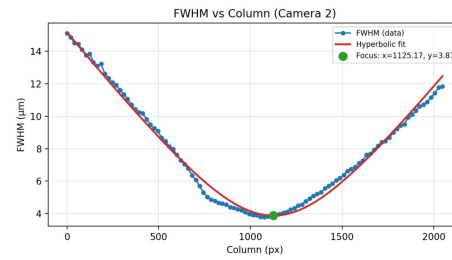
Camera snapshot + overlays (Camera 1):



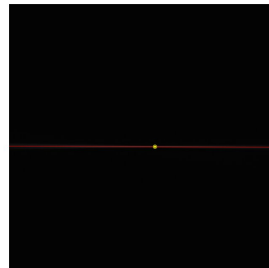
(B) Page 2 of the QC report.

Results - Camera 2

Angle Tilt (deg): 0.233
 Focus (col,row): (1125,1100.09)
 Beam Waist (FWHM at focus): 3.872 μm
 FWHM graph:



Camera snapshot + overlays (Camera 2):



(c) Page 3 of the QC report.

FIGURE F.1: Example of a Quality Control report for a LS beam measured at the centre of the FOV using fluorescein and a GFP emission filter.

G. External Resources

G.1. Software github repository

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

<https://github.com/diogojobf21/Development-Characterisation-and-Debugging-of-a-custom-Light-Sheet-Fluorescence-Microscope/tree/main/software>

G.2. Camera 1 vs Camera 2 beam tilt data

For Fluorescein, the data and QC report of beam tilt along light sheet between Camera 1 and Camera 2 is available at following repository:

https://github.com/diogojobf21/Development-Characterisation-and-Debugging-of-a-custom-Light-Sheet-Fluorescence-Microscope/tree/main/Fluorescein_data

G.3. Beam Characterisation Data

All results obtained were compiled in an Excel spreadsheet available at the following repository:

https://github.com/diogojobf21/Development-Characterisation-and-Debugging-of-a-custom-Light-Sheet-Fluorescence-Microscope/blob/main/Beam_characterization.xlsx

H. Example of a MetroloJ_QC field-illumination analysis in widefield mode

This appendix presents an example of a field-illumination analysis performed with the MetroloJ_QC plugin for the Nile Blue dataset acquired in Widefield Mode. This example complements the Confocal Mode analysis presented in Section 4.4, allowing direct comparison between the illumination uniformity obtained in both acquisition modes.

The image shown in Figure H.1 corresponds to the field-illumination pattern generated by MetroloJ_QC for the 640 nm excitation condition using Nile Blue. As discussed in Section 4.4, this condition presented the lowest field-uniformity value among the tested wavelengths, making it a representative example of the most challenging illumination case.

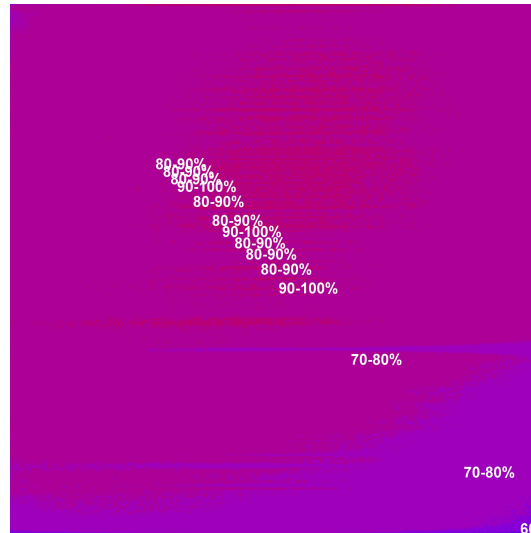


FIGURE H.1: Example of a MetroloJ_QC field-illumination analysis for the Nile Blue dataset acquired in Widefield Mode. The image corresponds to the 640 nm excitation condition and illustrates the spatial illumination pattern used to calculate the field-uniformity value reported in Section 4.4.