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Oblique Plane Microscopy using Dynamic Remote Focusing

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Abstract

Light-sheet microscopy has become a powerful technique for imaging biological samples across microscopic and mesoscopic scales because of its low phototoxicity, intrinsic optical sectioning, and large field of view (FoV). However, conventional light-sheet systems typically employ two orthogonally arranged objectives with limited working distances, which restricts their ability to image conventionally mounted samples. Oblique plane microscopy (OPM) overcomes this limitation by using a single objective for both illumination and detection, enabling light-sheet imaging of standard sample geometries. Since the same objective is used for both excitation and detection, the light sheet must be introduced obliquely with respect to the optical axis. Imaging this tilted plane is inherently challenging, as planes that are not coincident with the focal plane of the objective experience defocus across the field of view. Even though this approach retains all the fundamental advantages of light-sheet microscopy, the tilted remote-focusing geometry results in partial clipping of the primary objective's aperture. This reduces the effective numerical aperture (NA) and consequently degrades spatial resolution. To address this limitation, a full-aperture oblique-plane microscope was developed using off-the-shelf optical components to preserve the full detection aperture of the imaging objective.

In this thesis, a full-aperture oblique-plane microscope was implemented using dynamic remote focusing instead of the conventional relay-based remote-refocusing arrangement. This approach eliminated the tilted detection geometry by implementing dynamic remote focusing with an electrically tunable lens (ETL). The ETL is a current-controlled optical element whose optical power varies in proportion to the applied input current within its operating range. By modulating the driving current, the axial focal position can be changed electronically without any mechanical translation of the lens. This dynamic remote focusing, combined with synchronized rolling-shutter detection, enables accurate capture of the illuminated oblique plane without defocus. Following initial validation of the system, the microscope was upgraded by integrating a galvanometric mirror scanner to enable

faster volumetric imaging. In addition, a wavelength-splitting module was incorporated into the detection pathway, together with an additional excitation channel, to enable dual-colour imaging. These upgrades enabled the microscope to image a wider range of biological specimens, including samples exhibiting dynamic processes.

The performance of the microscope was evaluated at both low and high magnification using 20x/0.50 NA and 60x/1.10 NA objectives, respectively. The absence of tilted detection geometry allowed the objectives to be switched using the turret without requiring optical realignment, thereby enabling the same specimen to be imaged at different fields of view and spatial resolutions. The 20x configuration enabled large-field imaging of extended biological specimens, whereas the 60x configuration provided higher-resolution visualization of selected regions of interest. The versatility of the system was further demonstrated by imaging a range of prepared and living biological specimens, including rapid dynamic processes such as the beating zebrafish heart. In addition, an angular-spectrum-based simulation framework was implemented to model point-spread-function formation in the oblique imaging geometry and to provide a numerical estimate of the expected spatial resolution under the model's assumptions.

Chapter 1

1. A Review on oblique LSFM, with quantitative evaluation of the performances of different implementations

1.1. An introduction to fluorescence microscopy

Fluorescence microscopy has progressively gained a prominent position in biological and biomedical research over the past decades. This prominence stems from its ability to visualize specific cellular structures using fluorescent molecules, commonly referred to as fluorophores [1]. These molecules absorb light at defined excitation wavelengths and subsequently emit light at longer emission wavelengths. The resulting spectral separation between excitation and emission allows efficient discrimination of the fluorescence signal from background excitation light, thereby providing high imaging contrast [2].

Fluorescence imaging can rely either on endogenous fluorophores, which are naturally present within the specimen, or on exogenous fluorophores introduced as labelling agents. Since many biological structures do not exhibit sufficient intrinsic fluorescence, exogenous fluorophores are often used to stain or label the specimen. These fluorophores can be engineered or chemically functionalized to bind selectively to target molecules within a sample, enabling accurate visualization of the spatial distribution of labelled components. Because their molecular dimensions are negligible compared to the wavelength of visible light, fluorophores can be approximated as point emitters, an assumption that forms the basis for modelling image formation in fluorescence microscopy. Furthermore, the availability of spectrally distinct fluorophores permits simultaneous labelling of multiple targets within complex biological specimens. This multiplex labelling capability allows the investigation of the spatial organization and interactions of several structures in parallel [1].

Fluorescence originates from electronic transitions between discrete molecular energy states. Upon absorption of a photon, a fluorophore is excited from the electronic ground state to a higher excited state. This excitation is typically followed by rapid vibrational relaxation and other non-radiative processes that dissipate part of the absorbed energy as heat. Consequently, the emitted photon possesses lower energy and therefore a longer wavelength than the excitation light. The difference between excitation and emission wavelengths is referred to as the Stokes shift (fig. 2a). A finite Stokes shift is essential for fluorescence microscopy, as it enables effective spectral separation of the emitted fluorescence from the excitation light, thereby improving signal-to-noise ratio and detection sensitivity [1], [2].

During the excited-state lifetime, fluorophores may also undergo additional non-radiative pathways, including internal conversion and intersystem crossing. These processes convert electronic energy into vibrational energy, which is transferred to the surrounding medium as heat (fig. 2b). At high excitation intensities, such energy dissipation can lead to local heating and the formation of reactive chemical species, potentially resulting in photodamage to the biological specimen [1], [2].

Prolonged or repeated illumination may further induce irreversible photochemical modifications of the fluorophore, causing a permanent loss of fluorescence. This phenomenon, known as photobleaching, reduces signal intensity over time and imposes a fundamental limitation on imaging duration and long-term live-cell experiments [2], [3].

One of the simplest and most widely used implementations of fluorescence microscopy is widefield or epifluorescence microscopy (fig. 2c), in which the entire field of view is illuminated simultaneously. Although spectral filtering enables discrimination between excitation and emission wavelengths, widefield illumination excites fluorophores throughout the entire sample volume. Consequently, fluorescence originating from out-of-focus planes is also collected by the detector, resulting in increased background signal, reduced image contrast, and poor optical sectioning capability (fig. 1, panel 1). These limitations restrict imaging performance, particularly in thick or highly scattering biological specimens [3].

To overcome these drawbacks, optically sectioned fluorescence imaging modalities were developed, most notably Confocal microscopy and Two-photon microscopy. Confocal microscopy employs point illumination combined with a spatial pinhole to reject out-of-focus fluorescence, thereby improving axial resolution and contrast (fig.1, panel 2). Owing to its ability to provide optical sectioning and three-dimensional reconstruction, confocal microscopy became a gold standard technique for fluorescence imaging of moderately thick samples [4], [5].

Two-photon microscopy further enhances optical sectioning through nonlinear excitation, in which fluorophores are excited only at the focal volume by the near-simultaneous absorption of two lower-energy photons. The use of longer excitation wavelengths reduces scattering and enables deeper tissue penetration, making this technique particularly well suited for imaging thick and highly scattering biological samples [2], [5].

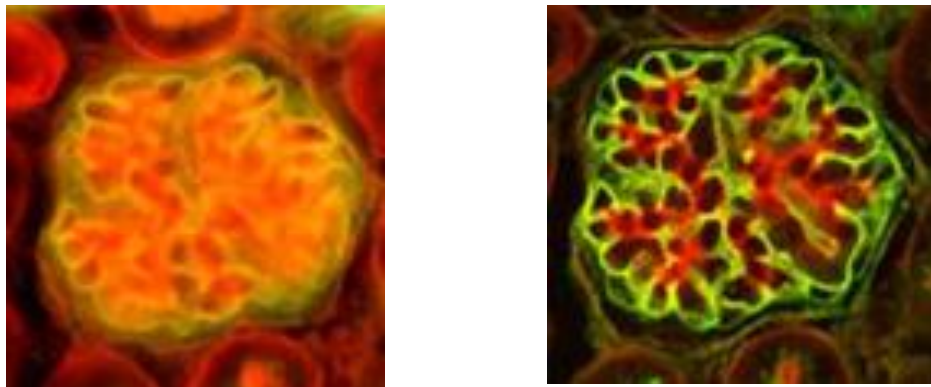


Figure 1. Comparison of a widefield fluorescence image of a thick sample, showing substantial out-of-focus background, with an optically sectioned confocal image showing improved contrast and suppression of out-of-focus fluorescence [6].

Despite these advances, both confocal and two-photon microscopy rely on intense, localized illumination to generate sufficient fluorescence signal. The high photon flux can accelerate photobleaching and induce phototoxic effects through local heating and the generation of reactive chemical species. These effects may perturb normal cellular physiology and ultimately limit imaging duration, especially in long-term or live-cell experiments [2], [5].

These limitations necessitated the development of more advanced imaging techniques for biological specimens, ultimately leading to the invention of light-sheet

fluorescence microscopy. Light-sheet microscopy combines intrinsic optical sectioning with selective plane illumination, enabling high-contrast, three-dimensional imaging while minimizing photodamage and photobleaching, a critical requirement for long-term observation of living samples [3], [7].

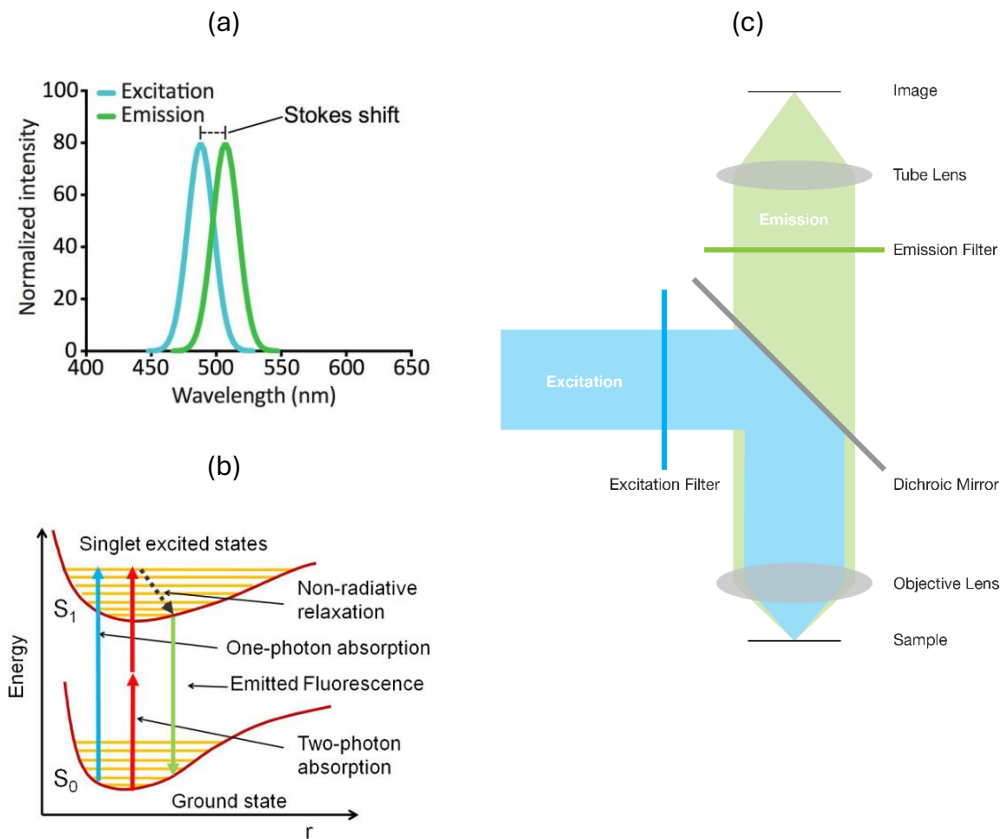


Figure 2. a) Plot showing excitation and emission with red shift in fluorescence. b) Energy-level diagram of a fluorophore showing vibrational energy states and the associated radiative and non-radiative relaxation pathways. c) Schematic of a standard epi-fluorescence microscope, in which the illumination and detection paths are separated by a dichroic mirror [2].

1.1 Light sheet fluorescence microscopy

Light-sheet fluorescence microscopy (LSFM) is an advanced imaging technique that achieves optical sectioning while minimizing photodamage to the sample. In a typical LSFM setup (fig. 3), two objectives are arranged orthogonally: one for illumination and the other for detection. The illumination objective generates a thin sheet of light—either by shaping the beam with a cylindrical lens or by rapidly scanning a focused beam—to selectively excite a single plane of the fluorescently labelled sample. The

detection objective, positioned perpendicular to the illumination plane, collects the emitted fluorescence from the entire illuminated plane, effectively performing widefield detection [2], [7].

Because only the imaged plane is illuminated at any given time, light-sheet microscopy reduces the total excitation light delivered to the sample. This selective plane illumination minimizes photobleaching and phototoxicity, making LSM particularly well suited for long-term imaging of living specimens while producing high-contrast, optically sectioned images [3].

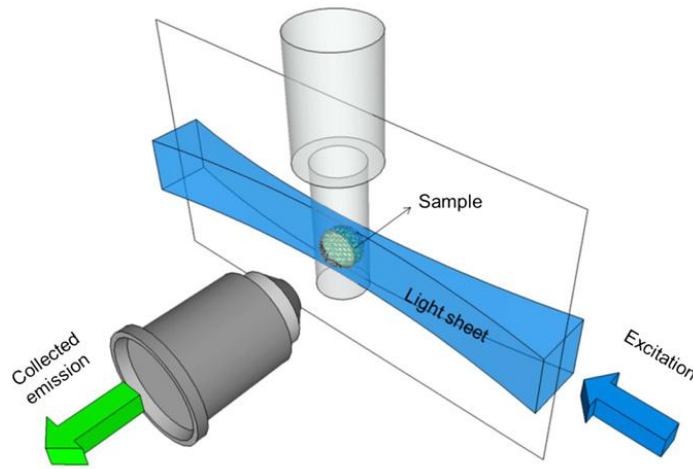


Figure 3. Schematic showing the basic optical imaging configuration in light sheet microscopy [2].

Ideally, light-sheet microscopy illuminates a single plane within the sample; however, in practice, it is not possible to confine the illumination perfectly to an infinitely thin plane. The light sheet has a finite thickness, which determines the volume of the sample that is excited at any given time. This thickness directly governs the axial resolution, or optical sectioning capability, of the light-sheet microscope. Importantly, the thickness of the illumination is strongly dependent on the numerical aperture (NA) of the illumination objective: higher NA produces a thinner light sheet and thus better axial sectioning, while lower NA results in a thicker sheet and reduced sectioning performance. Eq. (1) defines the equation for beam waist (w_0) for a Gaussian light sheet, while Eq. (2) gives the corresponding light-sheet thickness [8].

$$w_0 = \frac{\lambda_{exc}}{\pi NA_{ill}} \quad (1)$$

$$z_{LS} = 2w_0 = \frac{2\lambda_{exc}}{\pi NA_{ill}} \quad (2)$$

where λ_{exc} is the excitation wavelength and NA_{ill} is the illumination numerical aperture.

In addition to determining the light-sheet thickness, the NA of the illumination governs the beam divergence. A higher NA results in a highly divergent beam, which limits the distance over which the light maintains a thin, sheet-like profile. Conversely, a lower NA produces a less divergent beam, allowing the light sheet to extend over a longer distance. For a Gaussian beam, this propagation distance is characterised by the Rayleigh range x_R ,

$$x_R = \frac{\pi w_0^2}{\lambda_{exc}} \quad (3)$$

The Rayleigh range can also be expressed as,

$$x_R = \frac{\lambda_{exc}}{\pi NA_{ill}^2} \quad (4)$$

The usable light-sheet field of view along the propagation direction can be approximated by the confocal parameter,

$$FoV_{LS} \approx 2x_R \quad (5)$$

$$FoV_{LS} \propto \frac{1}{NA_{ill}^2} \quad (6)$$

Hence this divergence directly determines the effective FoV that the detection objective can image while maintaining acceptable optical sectioning performance. And from Eq. (6) the FoV is inversely proportional to the square of the NA_{ill} . Therefore, the choice of NA and the consequent trade-off between light-sheet thickness and usable FoV, is decided based on the specific application at hand [2],[8].

The lateral resolution in light-sheet microscopy is determined primarily by the detection objective, while the axial sectioning performance is strongly influenced by the thickness of the illumination light sheet. For a diffraction-limited detection

objective, the lateral resolution (r) can be approximated using the Rayleigh criterion as,

$$r = \frac{0.61\lambda_{em}}{NA_{det}} \quad (7)$$

Where λ_{em} is the emission wavelength and NA_{det} is the numerical aperture of the detection objective. The overall, or effective, resolution of the system is given by the product of the Point Spread Functions (PSFs) of the illumination and detection paths, reflecting the combined contributions of both objectives to image formation [8].

In light-sheet microscopy, the NA of the objectives plays a critical role in determining both optical performance and practical system design. Ideally, both the detection and illumination objectives would have high NA to maximize lateral and axial resolution. However, using high-NA objectives for both paths introduces geometric hindrance (fig. 4), which limits the physical space available for sample mounting and alignment.

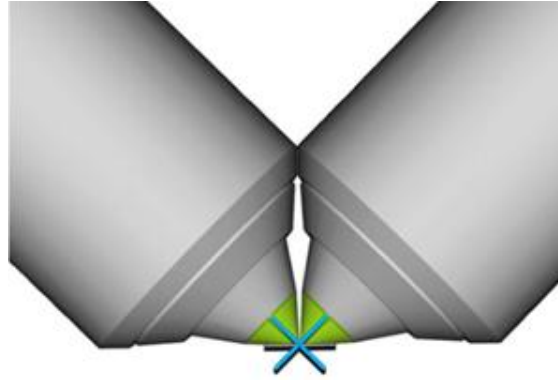


Figure 4. Schematic illustrating the spatial constraints imposed by the use of high-NA objectives for both illumination and detection in light-sheet microscopy [2].

This necessitates specialized sample mounting strategies and custom imaging chambers, often filled with imaging media, to accommodate the orthogonal arrangement of the objectives while maintaining optical access to the specimen. Such constraints not only restrict the choice of objectives but also limit the dimensions and shape of samples that can be imaged. Moreover, volumetric imaging requires scanning the sample through the light sheet, which can be highly restricted in LSM due to these geometric and mechanical limitations. In practice, the

detection objective is generally chosen to have a high NA to achieve optimal lateral resolution, while the illumination objective often employs a lower NA to extend the working distance and provide sufficient clearance between the objectives. Although a lower illumination NA produces a slightly thicker light sheet, this compromise allows the system to illuminate a large field of view while maintaining adequate optical sectioning. Consequently, the design of a light-sheet microscope involves balancing the high-resolution requirements of the detection path with practical constraints imposed by illumination geometry, working distance, and sample accessibility. This is precisely where oblique plane microscopy (OPM) finds its place [2], [8], [9].

1.2 An overview of oblique light sheet microscopy

Oblique plane microscopy (OPM) utilizes a light sheet to illuminate the sample, and the fluorescence emission is collected by the same objective that is used for illumination. This single-objective design overcomes many of the geometric and sample-mounting constraints inherent to conventional orthogonal light-sheet microscopy. By combining the advantages of selective plane illumination with a simplified geometry, OPM enables high-resolution, optically sectioned imaging of larger and more complex specimens while minimizing photodamage and retaining the key benefits of conventional light-sheet microscopy [9].

Although Oblique plane microscopy (OPM) employs a single objective for both illumination and detection, this configuration introduces its own set of challenges. Because the same objective must simultaneously deliver the excitation light and collect the emitted fluorescence, the illumination cannot be aligned axially. Axial illumination would confine excitation to a narrow region along the optical axis, resulting in the detection of only a small area on the camera and preventing visualization of the extended region within the Rayleigh range. Furthermore, purely axial illumination would fail to provide optical sectioning, which is a defining advantage of light-sheet-based imaging. To address this limitation, the excitation beam is introduced at an oblique angle emerging from the edge of the objective lens,

generating a tilted light sheet that intersects the focal plane and enables planar excitation while remaining compatible with single-objective detection [8], [10].

When the sample is illuminated obliquely, the excited plane is no longer coincident with the focal plane of the objective. Consequently, the illuminated plane appears tilted with respect to the detection focal plane, and fluorescence originating from different regions of the illuminated sheet is not simultaneously in focus. As a result, a conventional imaging configuration would introduce defocus across the field of view except around the focal plane, degrading image sharpness and optical sectioning performance. This geometric mismatch necessitates additional optical strategies, such as remote refocusing, to accurately image the oblique plane onto the camera [9], [11].

Direct detection of an oblique plane by mechanically tilting the camera or image sensor is, in principle, a simple geometric solution. However, such an approach is not suitable for high-numerical-aperture fluorescence microscopy. At high NA, imaging a tilted plane by just tilting the detector won't be enough to capture the oblique plane stigmatically. Although this may align the detector geometrically with the tilted image plane, different points along the oblique plane are imaged with different effective magnifications, leading to distortion and non-uniform sampling. In addition, microscope objectives are optimized to image a focal plane perpendicular to the optical axis, not an extended tilted plane. As a consequence, significant aberrations, including spherical aberration, astigmatism, and field-dependent distortions, are introduced into the imaging path. These aberrations deform the point spread function and degrade the diffraction-limited performance required for high-resolution biological imaging. Therefore, direct sensor tilting cannot provide accurate or quantitative optical sectioning [12], [13].

To address these limitations, Dunsby [9] proposed a method that combines oblique illumination with remote refocusing to enable aberration-free imaging of tilted planes (fig. 5). Inspired in part by the inclined illumination geometry of Highly inclined and laminated optical sheet microscopy, this approach introduces the excitation light into the sample at an oblique angle to generate a thin, optically sectioned fluorescence plane. Instead of directly detecting this plane with a tilted sensor, the

emitted fluorescence is first relayed to a remote image space using a secondary objective arranged in a pupil-conjugate configuration with the primary objective. This relay system faithfully reproduces the three-dimensional light distribution of the sample while preserving magnification, numerical aperture, and wavefront integrity [9], [13].

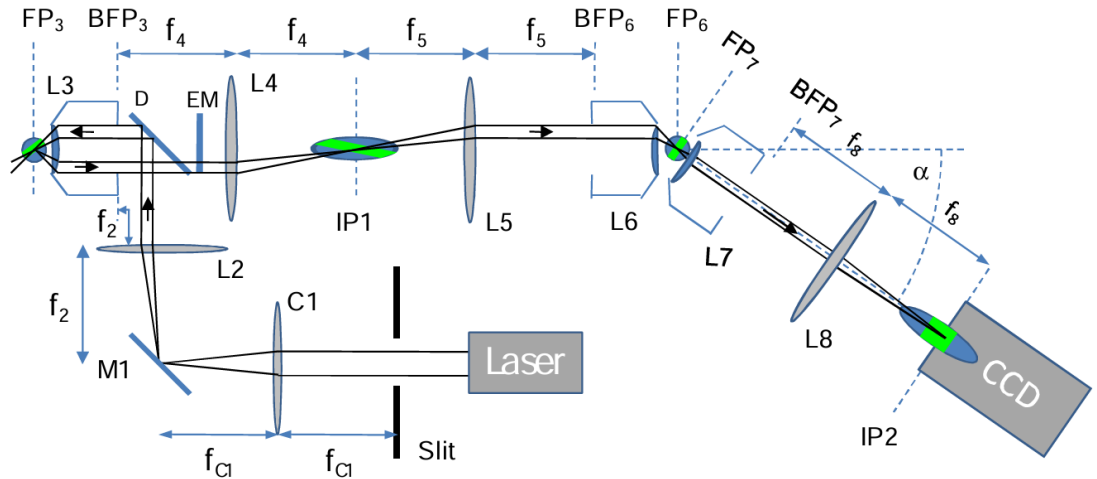


Figure 5. Schematic of a conventional oblique plane microscopy (OPM) [9].

Once the optical field is reconstructed in the remote space, a tertiary objective observes this intermediate image from a tilted orientation such that the entire oblique plane is conjugate to the focal plane of the detection camera. Because the tilt is introduced in the re-imaged space rather than at the primary image plane, the system maintains diffraction-limited performance while avoiding the aberrations associated with direct detector tilting. Consequently, the full illuminated plane can be imaged sharply and simultaneously, enabling true optical sectioning with high contrast and allowing structures outside the nominal focal plane of the primary objective to be visualized. [9], [14].

This remote-refocusing strategy combined with the tilted imaging forms the foundation of Oblique plane microscopy, which achieves light-sheet-like sectioning and reduced photodamage while utilizing only a single primary objective for both illumination and detection [9].

While remote refocusing enables aberration-corrected detection of oblique planes and forms the basis of Oblique plane microscopy (OPM), the introduction of multiple relay objectives and tilted detection geometry gives rise to additional practical and optical constraints. In particular, the oblique re-imaging configuration inherently leads to an effective mismatch between the numerical apertures of the primary detection objective and the downstream relay optics (fig. 6). This mismatch results in incomplete transmission of the collected fluorescence and consequently limits the achievable spatial resolution [11], [15]. This is one of the major limitations of the conventional OPM system.

1.3 Limitations of oblique light sheet microscopy

In an ideal diffraction-limited microscope, the full pupil of the detection objective is relayed to the image plane without loss, thereby preserving the entire angular spectrum of the emitted light. Under such conditions, all spatial frequencies supported by the objective contribute to image formation, and the system achieves its theoretical resolution. In OPM, however, the fluorescence collected by the primary objective must be redirected through a remote refocusing system and subsequently observed by a tertiary objective at an oblique angle. Owing to the finite acceptance angle of the tertiary objective and the geometric constraints imposed by the tilted configuration, the emission cone from the primary objective cannot be completely captured by the tertiary objective. As a result, only a fraction of the pupil is transmitted through the relay system, leading to partial clipping or truncation of the effective aperture [15], [16].

From a Fourier-optical perspective, image formation is governed by the pupil function, and the point spread function (PSF) corresponds to the squared magnitude of the Fourier transform of the pupil function. Consequently, truncation of the pupil removes high spatial frequency components and reduces the effective detection NA, thereby broadening the PSF and degrading resolution. Moreover, because the clipping introduced by the oblique geometry is typically asymmetric, the resulting pupil becomes anisotropic. This anisotropy produces direction-dependent resolution and manifests as elongation of the PSF along the axis corresponding to the missing

spatial frequencies. Experimentally, this appears as reduced contrast and a stretched focal spot, particularly along the oblique or axial direction [11], [14], [17].

Therefore, the resolution in OPM is not determined solely by the nominal NA of the primary objective, but rather by the overlap of the pupils of all objectives within the relay system. Careful matching of magnification, numerical aperture, and refractive index between the primary, secondary, and tertiary objectives can mitigate these losses; nevertheless, complete pupil transmission is difficult to achieve in practice due to geometrical constraints and limited angular acceptance. This trade-off represents a fundamental limitation of OPM: although the technique simplifies the sample mounting constraints and preserves the low-phototoxicity advantages of light-sheet illumination, it does so at the expense of reduced optical throughput and modest anisotropic resolution loss [11].

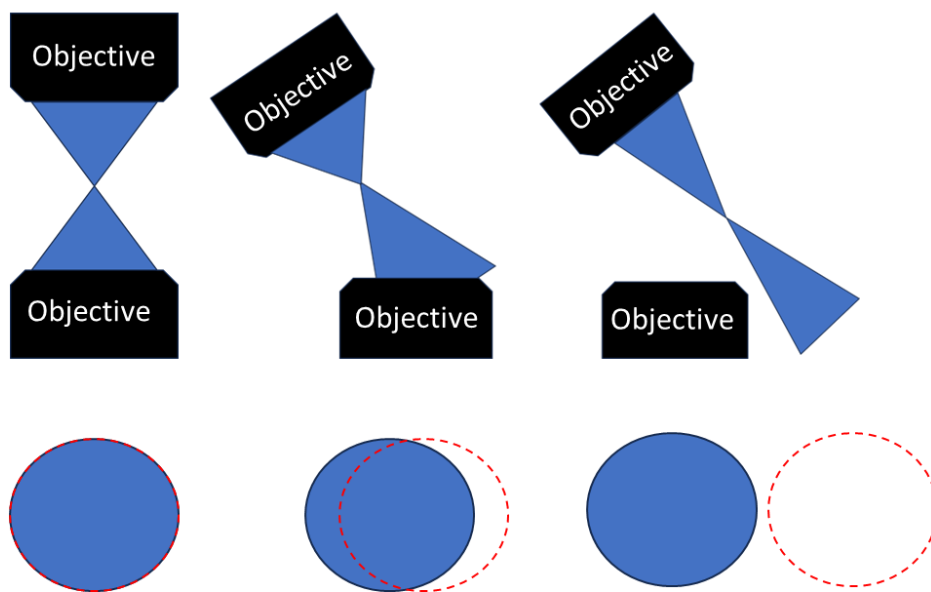


Figure 6. Schematic illustrating the progressive loss of collected light at the tertiary objective for different objective tilt angles..

In oblique plane microscopy, the choice of NA for the primary objective plays a critical role not only in determining lateral and axial resolution but also in defining the allowable oblique angles of the illumination light sheet. Objectives with low NA inherently restrict the maximum cone angle of the excitation beam, which limits the tilt of the illumination plane that can be generated within the sample. Because the emitted fluorescence from the obliquely illuminated plane must subsequently be relayed through the remote refocusing system, the angular extent of the emission

cone is directly constrained by the NA of the primary objective. A limited cone angle reduces the overlap with the acceptance cone of the tertiary objective at the remote focus plane, exacerbating pupil truncation and further restricting the effective detection NA. Consequently, low-NA objectives are generally unsuitable for high-resolution OPM, as they not only reduce the achievable spatial resolution but also limit the optical sectioning capability and the fidelity with which the oblique plane can be imaged [10], [18].

However, the use of high-NA objectives inherently restricts the field of view, limiting the lateral extent of the sample that can be imaged in a single acquisition. This trade-off illustrates a fundamental constraint of OPM: achieving high-resolution oblique-plane imaging and adequate pupil coverage necessitates high NA, but doing so simultaneously reduces the accessible imaging volume, particularly for applications requiring mesoscopic-scale coverage. This resulted in the need for newer variants of OPM [15], [18], [19].

1.4 Alternative methods for OPM

To mitigate the issue of missing aperture in oblique plane microscopy, several strategies have been developed to improve the overlap between the angular extent of the fluorescence emission and the acceptance cone of the tertiary objective in the remote refocusing system. One effective approach involves introducing a refractive index discontinuity at the remote image space to reduce the angular spread of the emission as it transitions between media (fig. 7c). This angular compression allows the tertiary objective to capture a larger fraction of the emitted light. A widely adopted implementation of this concept is the use of a glass-tipped tertiary objective in the remote space. By imaging through a high-refractive-index medium, this configuration effectively increases the acceptance angle of the tertiary objective, typically achieving an effective NA approaching unity [16], [20].

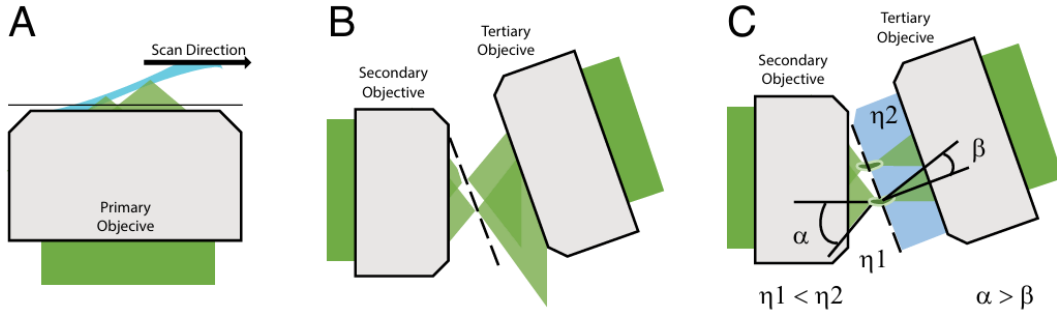


Figure 7. Schematic illustrating different mechanisms associated with light collection in oblique illumination. (a) Oblique illumination geometry. (b) Part of the emitted light cone falls outside the collection aperture of the objective (missing cone). (c) A refractive-index mismatch at the interface causes refraction and compression of the light cone, allowing capture of full cone of light [16].

However, the maximum usable angle is fundamentally limited by the critical angle at the refractive index interface. Emission rays exceeding the critical angle undergo total internal reflection and cannot be transmitted into the subsequent optical medium. Consequently, although refractive index engineering can significantly improve pupil overlap and reduce aperture truncation, complete recovery of all high-angle components is physically constrained by total internal reflection [16].

By improving pupil overlap between the primary and tertiary objectives within these physical limits, this design reduces effective aperture loss and mitigates anisotropic elongation of the point spread function, leading to improved and more symmetric resolution in oblique plane microscopy.

An alternative strategy to mitigate aperture mismatch in oblique plane microscopy involves the introduction of a diffraction grating at the remote image space (fig. 8). Rather than relying solely on high-NA tertiary objectives or refractive index discontinuities to preserve the angular spectrum, this approach redistributes the spatial frequency content of the emitted fluorescence through diffraction. Specifically, the grating diffracts high-angle emission components into directions that fall within the acceptance cone of the tertiary objective. As a result, spatial frequencies that would otherwise be lost due to pupil truncation can be partially recovered.

This method enables the use of lower-NA primary objectives, which inherently provide a larger field of view. By redirecting angular information at the remote plane,

the diffraction grating relaxes the strict requirement for extremely high NA in the primary objective, thereby reducing geometric constraints and expanding the accessible imaging volume. However, this approach introduces its own trade-offs, including diffraction efficiency losses, wavelength dependence, and potential overlap of diffraction orders, which must be carefully managed in system design.

Consequently, diffraction-based angular redistribution offers a complementary solution to refractive index-based pupil compression methods, allowing improved balance between resolution, pupil coverage, and field of view in OPM systems [15].

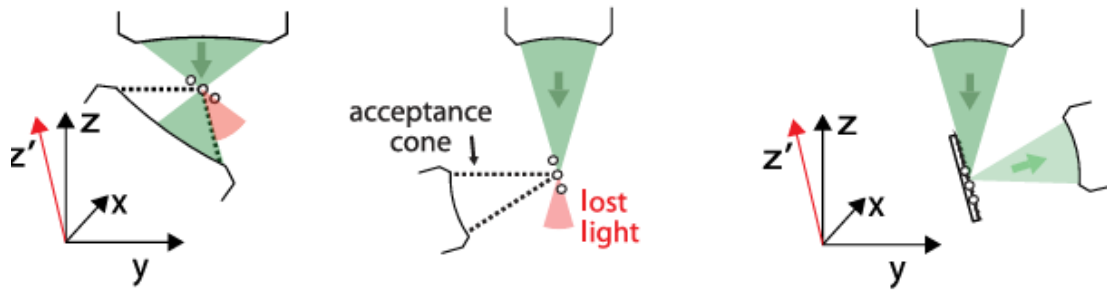


Figure 8. Schematic illustrating aperture mismatch in high-NA, limited-FoV and low-NA, large-FoV objective configurations. The reflective diffraction grating redirects otherwise uncollected light cone into the objective aperture, increasing the fraction of the light cone that can be captured [15].

1.5 Comparison of different OPM with simulation

Based on the different OPM architectures discussed earlier, a comparison was made using key optical and performance parameters, including objective numerical aperture, effective detection NA, spatial resolution, field of view, imaging volume, and acquisition rate. The comparison includes the original OPM implementation [9], a high-resolution glass-tipped tertiary objective design that preserves a larger fraction of the primary objective aperture [16], and a low-NA Diffractive OPM (DOPM) configuration designed for large-field volumetric imaging [15]. These systems represent different design regimes within OPM, ranging from aperture-limited high-resolution imaging to large-volume mesoscopic imaging (Table 1).

Parameter	OPM	Glass-tipped OPM	DOPM
Primary Objective	60x / 1.35 NA (oil)	100x / 1.35 NA (oil)	4x / 0.28 NA
Secondary Objective	40x / 0.85 NA (air)	40x / 0.95 NA (air)	4x / 0.28 NA
Tertiary Objective	10x / 0.3 NA (air)	Glass-tipped Objective	4x / 0.28 NA
Effective Detection NA	~0.45 (aperture limited)	Near-full NA is preserved	0.28
Lateral Resolution (FWHM)	~0.8 μm	~300 nm	~1–2 μm
Axial Resolution (FWHM)	~4.3 μm	~0.6–0.9 μm	~37 μm
Lateral FoV	~100–200 μm	~180 x 180 μm^2	~3.3 x 3.0 mm^2
Imaging Volume	~190 x 80 x 50 μm^3	~180 x 180 x 60 μm^3	~3.3 x 3.0 x 1.0 mm^3
Plane Acquisition Rate	Limited	~800 planes/s	Optimized for large-volume scanning
Design Emphasis	Concept demonstration	High-resolution subcellular imaging	Mesoscopic large-scale imaging

Table 1. Comparison of different types of OPM in the specific implementation reported in Ref [9], [15], [16]

Following this comparison, two representative high-resolution OPM configurations were selected for PSF simulation: the original OPM architecture and the glass-tipped tertiary objective architecture. These systems were chosen because the simulation study focused on resolution and PSF formation, rather than field of view or imaging volume. The original OPM configuration represents an aperture-limited case, whereas the glass-tipped tertiary objective configuration represents a design in

which a larger fraction of the primary objective aperture is preserved. The DOPM configuration was included in the comparison table to illustrate a low-NA, large-field imaging regime, but it was not included in the PSF simulation analysis because the present simulations were concerned with diffraction-limited resolution rather than field-of-view optimization.

1.5.1 Motivation for diffraction-based PSF simulation

Characterization of the optical performance of a microscope is a critical step in biological imaging, particularly when comparing systems with different optical architectures. Among the different metrics used to evaluate microscope performance, the point spread function (PSF) is a fundamental descriptor because it describes how an ideal point emitter is imaged by the system. The shape and extent of the three-dimensional PSF provide direct information about lateral resolution, axial resolution, and optical sectioning capability [21].

In OPM, PSF characterisation is especially important because the final imaging performance is not determined by the primary objective alone. It also depends on the effective pupil transmitted through the remote imaging system and on whether high-angle rays are preserved or truncated by the downstream optics. The three-dimensional PSF is closely related to the achievable lateral and axial resolution of the microscope and provides a quantitative description of how light from a point emitter is distributed in three dimensions. In optical sectioning microscopy, the axial extent of the effective system response is particularly important, because it influences background rejection, image contrast, and depth discrimination. Theoretical simulation of the PSF therefore provides a useful way to estimate diffraction-limited resolution and sectioning behaviour before experimental validation, and to compare how different OPM architectures influence the expected three-dimensional imaging response.

In this chapter, the simulation analysis is used to examine the resolution and PSF structure of the OPM architectures discussed above. The emphasis is placed on how effective numerical aperture, pupil transmission, and aperture truncation influence the predicted lateral and axial extent of the PSF. Other performance aspects, such as

field of view, imaging volume, acquisition speed, and camera readout strategy, are important for practical microscope design, but they are not the main focus of the present simulation analysis .

Several methods are available for simulating microscope PSFs, ranging from rigorous vector diffraction models to more computationally efficient scalar diffraction approaches. Accurate PSF modelling requires an appropriate treatment of diffraction, since the finite size and three-dimensional structure of the focal spot arise from wave propagation through the objective pupil .

The most rigorous description is provided by vector diffraction theory, which accounts for the electromagnetic nature of light, including polarisation effects, longitudinal field components, and coupling between field components. Vector models are particularly important for high-numerical-aperture systems, where large propagation angles contribute significantly to the focused field. However, vector diffraction theory is mathematically more complex and computationally demanding. Therefore, for many practical simulations, scalar diffraction theory is used as an approximation to describe the main diffraction-limited structure of the PSF [21].

Within scalar diffraction theory, the Rayleigh–Sommerfeld diffraction integral provides one of the most rigorous formulations of free-space propagation. It does not rely on the paraxial approximation and can therefore describe propagation beyond the small-angle regime. In this formulation, the optical field is treated as a scalar quantity, and polarisation-dependent effects are neglected. Although this simplification limits its validity for strongly vectorial focusing conditions, the Rayleigh–Sommerfeld formulation provides an accurate scalar description for many diffraction problems. In general, the integral does not have a simple closed-form solution for arbitrary fields and must be evaluated either numerically or through suitable approximations [22], [23].

The Fresnel and Fraunhofer diffraction formulae are commonly used approximations within scalar diffraction theory. The Fresnel approximation assumes paraxial propagation, where the dominant propagation directions remain close to the optical axis and the propagation phase can be approximated by retaining only the quadratic transverse-coordinate terms. The Fraunhofer approximation corresponds to the

further far-field limit, in which the diffraction pattern can be related to the Fourier transform of the aperture field. These approximations are useful in many optical systems, but they are not generally suitable for accurate near-focus PSF modelling in high-NA microscopy, where large-angle propagation and three-dimensional field evolution around the focal plane must be considered [21], [24], [25].

Unlike the Fresnel approximation, the angular spectrum method does not require the paraxial approximation when the full Helmholtz propagator is used. It can therefore represent large-angle scalar propagation more accurately than Fresnel-based methods, within the limits of scalar diffraction theory. This is particularly useful for PSF simulation, where the optical field must be evaluated over short axial distances around the focal region.

The Rayleigh–Sommerfeld integral can also be evaluated directly using numerical quadrature methods, such as trapezoidal or Simpson integration. In principle, direct numerical integration can provide accurate scalar propagation results if the field, aperture, and observation region are sampled sufficiently. However, for three-dimensional PSF simulations it becomes computationally expensive, because the field must be evaluated over many points and many axial planes. It is also sensitive to sampling errors when rapidly varying phase terms or high spatial-frequency components are present [21], [22].

For these reasons, the angular spectrum method was used in this thesis as the main scalar propagation method for simulating the PSF of the selected OPM architectures. In this approach, the field distribution in a plane is decomposed into its spatial-frequency components using a Fourier transform. Each spatial-frequency component represents a plane-wave contribution and propagates independently by multiplication with the corresponding free-space propagator. When the full Helmholtz propagator is used, the angular spectrum method does not impose the paraxial approximation, while remaining computationally efficient for repeated propagation over multiple axial planes. For this reason, it was selected as the scalar propagation formalism for the OPM PSF simulations presented in this chapter [21].

1.5.2 Angular spectrum propagation

In the angular spectrum method, the complex optical field defined in an input plane is represented as a superposition of plane-wave components. If the field at the source plane is denoted by $g(x,y,0)$, its angular spectrum is obtained by a two-dimensional Fourier transform,

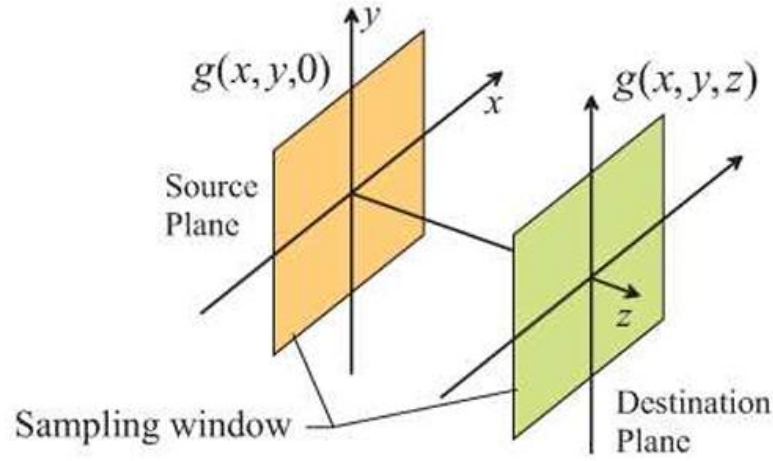


Figure 9. Definition of coordinate system and geometry of the angular spectrum mode used in the simulation [22].

$$G(k_x, k_y, 0) = F\{g(k_x, k_y, 0)\} \quad (8)$$

where k_x and k_y are the transverse components of the wavevector in the (x) and (y) directions, respectively. Each component of the angular spectrum corresponds to a plane wave with a particular transverse propagation direction. Propagation over an axial distance z is then calculated by multiplying the angular spectrum by the free-space transfer function $H(k_x, k_y)$ [22], [23], [25].

$$G(k_x, k_y; z) = G(k_x, k_y; 0) H(k_x, k_y; z) \quad (9)$$

where,

$$H(k_x, k_y) = e^{ik_z z} \quad (10)$$

and

$$k_z = \sqrt{k^2 - k_x^2 - k_y^2} \quad (11)$$

Here, $k=2\pi/\lambda$ is the wavenumber in a medium of refractive index n , and λ denotes the wavelength inside the medium. The propagated field is obtained by applying the inverse Fourier transform of $G(k_x, k_y; z)$ given by,

$$g(x, y; z) = F^{-1}\{G(k_x, k_y; z)\} \quad (12)$$

This formulation is convenient for PSF simulation because propagation is performed by multiplication in spatial-frequency space rather than by direct point-by-point evaluation of the Rayleigh–Sommerfeld diffraction integral. The coordinate system and geometrical conventions adopted in the angular spectrum simulation are illustrated in fig. 9, while the propagation of the angular spectrum between planes is shown schematically in fig. 10. When the full transfer function is used, the angular spectrum method does not require the paraxial approximation and can therefore represent large-angle scalar propagation more accurately than Fresnel-based propagation methods, within the limits of scalar diffraction theory.

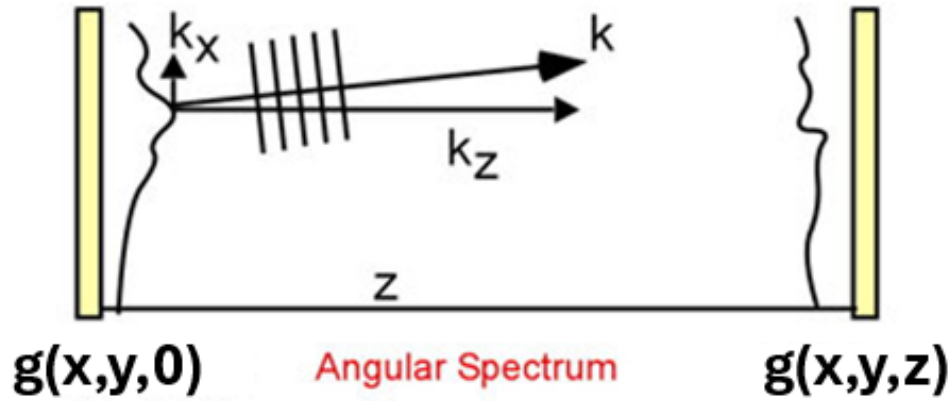


Figure 10. Schematic representation of diffraction of light modeled using angular spectrum propagation from reference plane to destination plane [26].

After defining the angular spectrum propagation formalism, the method was applied to simulate the detection PSF of the selected OPM architectures. In these simulations, the microscope objective was represented by its effective pupil function, which defines the spatial-frequency components transmitted by the optical system. The following sections describe how the original OPM configuration and the glass-tipped tertiary objective configuration were represented in the simulation, with

particular emphasis on the effective pupil support, aperture truncation, and their influence on the resulting detection and illumination PSFs [22].

1.5.3 Application of the angular spectrum method in OPM PSF simulation

In light-sheet fluorescence microscopy, the effective imaging response is determined by both the illumination and detection paths. So, the PSF of the entire light sheet microscope is given by the product of illumination and detection PSF. Therefore, in the present simulation, both the illumination PSF and the detection PSF were calculated separately before evaluating final psf of the system. In OPM, the detection path contains multiple objectives that relay the oblique image plane through a remote imaging system. The first part of this relay recreates the light distribution from the primary objective in a remote image space. That is in the OPM the first remote-imaging stage maps the focal region of the primary objective into a remote image space, where the oblique plane can be accessed by the downstream detection optics. Due to this in the current simulation the calculation was initiated in the remote image space of the secondary objective rather than directly at the sample [27].

The tertiary objective views the remote image space from an oblique angle. To represent this geometry, the angular spectrum in the remote space was rotated in spatial-frequency space into the coordinate frame of the tertiary objective. The rotated angular spectrum therefore defines the pupil distribution incident on the tertiary objective. Architecture-specific differences were then introduced through the tilt angle, the aperture support of the tertiary objective, and any additional collection constraints, such as pupil clipping or refractive-index-dependent acceptance in the glass-tipped configuration [9], [16], [25].

For the glass-tipped tertiary objective configuration, the remote image space was treated as air up to the focal region viewed by the tertiary objective. The refractive index of the glass tip was introduced only in the tertiary collection space. After the remote-space angular spectrum was rotated into the tertiary-objective frame, the higher refractive index of the glass was used to define the allowed k-space support

and the critical-angle transmission constraint of the effective tertiary pupil. The glass tip therefore modifies the collection geometry of the tertiary objective, rather than the propagation medium of the remote-space angular spectrum.

Although the main architecture-dependent comparison was based on the detection PSF, the illumination PSF was also calculated in the simulation. The illumination path was represented by a shifted slit pupil, corresponding to the finite aperture of the light sheet in the pupil plane. This slit was positioned according to the illumination angle and propagated using the same angular-spectrum formalism to obtain the three-dimensional illumination intensity distribution. The detection PSF and illumination PSF were then calculated separately and multiplied to obtain the effective system PSF under the incoherent fluorescence approximation [8].

This modelling strategy allowed the illumination, detection, and combined system PSFs to be calculated within a single scalar diffraction framework, while preserving the architecture-specific differences between the selected OPM configurations. The simulations therefore provide a basis for comparing how aperture truncation, pupil preservation, and tertiary-objective collection geometry influence the predicted lateral and axial resolution. The following section presents the simulated PSFs and uses them to evaluate the resolution performance of the selected OPM architectures [25].

1.5.4 Simulation results

Using the angular-spectrum-based simulation approach described above, the illumination, detection, and combined system PSFs were calculated for the selected OPM architectures. The results were analysed to compare how differences in effective pupil support influence the predicted resolution. Particular attention was given to the detection PSF, since aperture truncation in the downstream remote imaging optics directly modifies the transmitted spatial-frequency support and therefore affects the lateral and axial extent of the focal response.

For each configuration, the illumination PSF, detection PSF, and combined system PSF were visualized in orthogonal planes. The illumination PSF describes the finite thickness and tilted geometry of the light sheet, while the detection PSF describes

the response of the remote detection path. The combined system PSF was obtained by multiplying the illumination and detection intensity distributions, providing an estimate of the effective light-sheet imaging response under the incoherent fluorescence approximation.

The original OPM configuration represents an aperture-limited case. In this architecture, the downstream tertiary objective has a lower numerical aperture than the primary collection objective, so only a reduced portion of the primary objective pupil is transmitted through the complete detection path. This reduced effective pupil support removes high-angle spatial-frequency components and is expected to broaden the detection PSF, particularly along the axial direction.

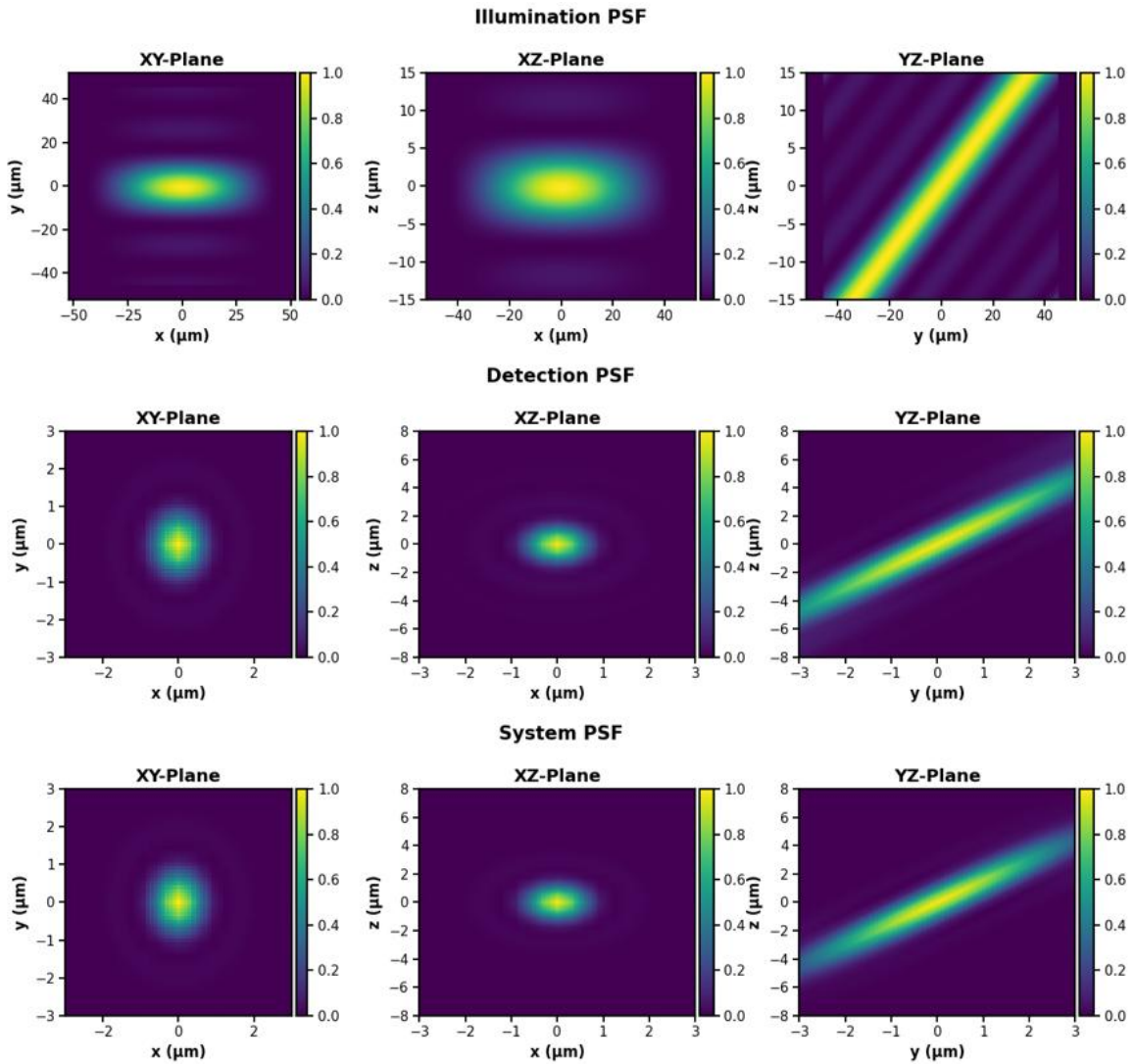


Figure 11. Simulated illumination, detection, and combined system PSFs for the original OPM configuration. The reduced effective pupil support caused by aperture limitation in the downstream detection optics broadens the detection response and contributes to an elongated axial PSF.

The glass-tipped tertiary objective configuration represents a higher-aperture OPM design in which a larger fraction of the primary objective pupil is preserved through the remote detection path. In this case, the tertiary collection geometry is designed to reduce the aperture truncation that limits the original OPM configuration. As a result, a larger range of high-angle spatial-frequency components contributes to image formation, leading to a more compact detection PSF and improved predicted resolution.

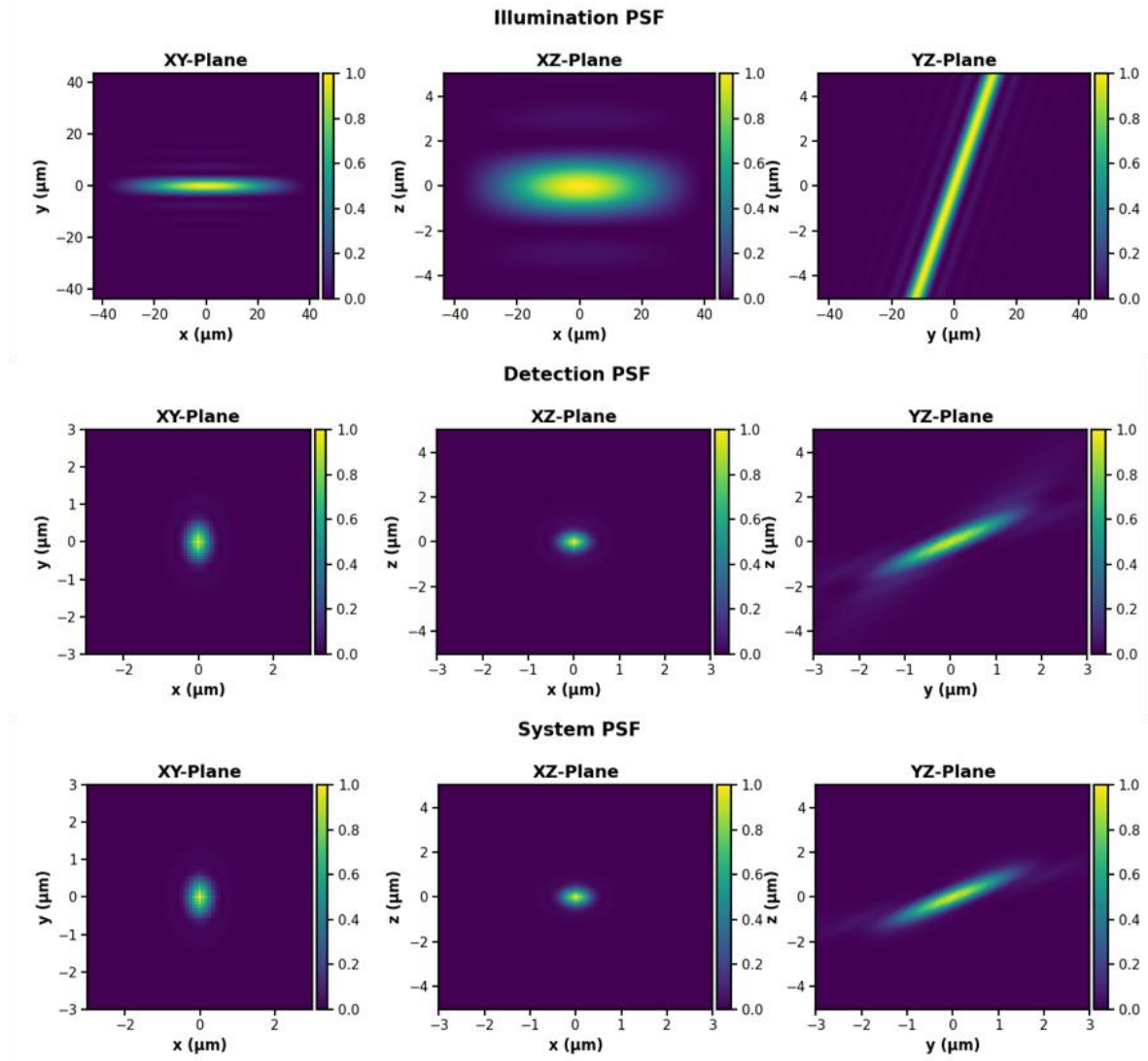


Figure 12. Simulated illumination, detection, and combined system PSFs for the glass-tipped tertiary objective OPM configuration. Compared with the aperture-limited original OPM case, the increased pupil support preserves a larger range of high-angle spatial-frequency components, resulting in a more compact detection response and improved predicted lateral and axial resolution.

Comparison of the two simulated configurations shows the importance of effective pupil support in OPM. In the aperture-limited configuration, truncation by the downstream detection optics reduces the transmitted spatial-frequency range and broadens the detection PSF. In contrast, the glass-tipped tertiary objective configuration preserves a larger fraction of the available pupil, producing a narrower detection response. This difference is reflected in the simulated lateral and axial FWHM (Full Width Half Maximum) values, demonstrating that the resolution of an OPM system is governed not only by the primary objective NA, but also by how effectively the remote imaging optics transmit the collected angular spectrum. The simulated illumination, detection, and combined system PSFs for the original and glass-tipped OPM configurations are shown in fig. 11 and fig. 12.

The simulated FWHM values show a clear difference between the two selected OPM architectures. For the original OPM configuration, the combined system PSF had FWHM values of approximately 700 nm along x, 800 nm along y, and 1.90 μm along z.

These values align perfectly with the reported lateral resolution of 800nm, but the axial resolution is overestimated compared with the reported 4.3 μm . This discrepancy can be due to the limitation of the simulation in recreating all experimental conditions, or in deviations from the diffraction limit of the reported experimental system (e.g. spherical aberration). In comparison, the glass-tipped tertiary objective configuration produced a more compact system PSF, with FWHM values of approximately 200 nm, 300 nm, and 600 nm along x, y, and z, respectively. This is also in agreement with the reported lateral resolution of 300 nm and axial resolution in the range of 600 to 900 nm. This improvement is consistent with the larger effective pupil support of the glass-tipped configuration, which preserves higher-angle spatial-frequency components that are truncated in the aperture-limited original OPM design.

The simulations were performed using the objective parameters summarised in Table 1 for the original OPM and glass-tipped tertiary objective configurations. In the original OPM configuration, the primary, secondary, and tertiary objectives were a 60x/1.35 NA oil-immersion objective, a 40x/0.85 NA air objective, and a 10x/0.3 NA air objective, respectively. In the glass-tipped OPM configuration, the corresponding

objectives were a 100x/1.35 NA oil-immersion objective, a 40x/0.95 NA air objective, and a custom glass-tipped tertiary objective. These two systems were selected because they represent different degrees of pupil preservation in the remote detection path. Therefore, the following discussion focuses on how their effective pupil support influences the simulated PSF and the resulting resolution estimates.

The simulated resolutions are consistent with the trends reported in the literature. For the original OPM configuration, the simulated lateral FWHM values are close to the reported sub-micron lateral resolution of the prototype system. For the glass-tipped tertiary objective configuration, the simulated PSF is substantially more compact and gives resolution values in the same range as the high-resolution OPM measurements reported by Sapoznik et al [16]. The comparison should be interpreted as qualitative and order-of-magnitude agreement, since the simulations use ideal scalar-diffraction assumptions and do not include all experimental factors, such as aberrations, bead size, alignment errors, noise, and the deconvolution procedure.

Overall, the simulation results support the conclusion that effective pupil support is a key factor determining the resolution performance of OPM systems. The original aperture-limited OPM configuration produces a broader PSF because the downstream detection optics transmit only a reduced portion of the collected angular spectrum. In contrast, the glass-tipped tertiary objective configuration preserves a larger fraction of the available pupil, leading to a more compact simulated PSF and improved predicted resolution. These results are consistent with reported performance trends in the literature and justify a diffraction-based simulation method for the full-aperture OPM approach developed later in this thesis.

The simulation framework developed here, in the next chapter addresses a different OPM implementation. Instead of physically rotating the detection plane to coincide with the oblique illumination plane, the system uses dynamic remote focusing to scan the detection focus and rolling-shutter synchronization to recover optical sectioning. Therefore, the Chapter 2 simulation does not use the same tilted tertiary-objective geometry as the conventional OPM comparison. Instead, it models the

coordinated behaviour of the illumination, detection focus, remote focusing, and rolling-shutter readout in the full-aperture system.

2. Full aperture oblique LSFM with remote focusing

2.1. Remote focusing technique.

The fundamental objective of a modern optical microscope is to reconstruct the three-dimensional structure of a specimen with high fidelity. For aberration-free imaging, the angular distribution of rays emerging from a point in object space must be mapped into image space while preserving the same angular distribution. Maxwell showed that perfect stigmatic imaging between two media of refractive indices n_1 (object space) and n_2 (image space) requires the transverse magnification M to satisfy ,

$$M = \frac{n_1}{n_2} \quad (13)$$

This condition ensures isotropic scaling of the electromagnetic field and preserves the angular relationships of rays across the interface. In practical microscopy, however, objectives are designed to provide much higher magnifications than n_1/n_2 , and therefore exact Maxwellian imaging is not achieved globally throughout three-dimensional space[12], [13].

Nevertheless, subsets of points can be imaged without aberration under specific conditions. Two classical conditions describe these cases:

- The Abbe sine condition, which ensures that points in a plane perpendicular to the optical axis are imaged perfectly.
- The Herschel condition, which ensures stigmatic imaging of axial points along the optical axis.

Modern microscope objectives are primarily designed to satisfy the Abbe sine condition, optimizing imaging performance for points located within the focal plane. Consequently, a single plane within the specimen can be imaged with high fidelity. However, points displaced axially from the focal plane experience increasing aberrations, primarily defocus and spherical aberration[13].

Therefore, volumetric imaging requires sequential acquisition of multiple planes. Conventionally, this is achieved by mechanically scanning the specimen through the focal plane. However, mechanical scanning can introduce vibration, drift, and other perturbation of delicate biological samples.

To overcome these limitations, remote focusing was introduced. The key idea is to decouple axial scanning from the physical specimen by recreating the optical field distribution of the sample in a remote space. This is achieved using a back-to-back objective configuration in which a secondary objective is placed pupil-conjugate to the primary objective. If the combined magnification of the relay system satisfies Maxwell's condition as mentioned earlier and if the magnification is isotropic, then the light distribution produced by the sample can be exactly reproduced in the remote space.

The phase at the back focal plane of the objective due to a point source located at an arbitrary axial position z can be expressed in terms of the normalized polar pupil coordinates (ρ, ϕ) where ρ is the normalised radial coordinate measured from the pupil centre and ϕ is the azimuthal angle as,

$$\Psi(\rho, \phi; \mathbf{r}) \approx nk \sin \alpha \left\{ x\rho \cos \phi + y\rho \sin \phi + z \sqrt{\frac{1}{\sin^2 \alpha} - \rho^2} \right\} \quad (14)$$

expanding the phase expression in the above expression as a Taylor series yields,

$$znk \sqrt{1 - \rho^2 \sin^2 \alpha} = znk \left\{ 1 - \frac{\rho^2 \sin^2 \alpha}{2} + \frac{\rho^4 \sin^4 \alpha}{8} + \dots \right\} \quad (15)$$

The second-order term corresponds to quadratic defocus, which can be compensated by refocusing through the tube lens for a given z . However, higher-order terms correspond to spherical aberration and cannot be corrected by simple refocusing. To tackle this issue the odd parity of the pupil function is exploited. That is

the pupil phase associated with a point source at position $+r$ is equal in magnitude and opposite in sign to that of a source at position $-r$, due to symmetry of the pupil function as shown in fig. 13. That is,

$$\Psi(\rho, \phi; -\mathbf{r}) = -\Psi(\rho, \phi; \mathbf{r}) \quad (16)$$

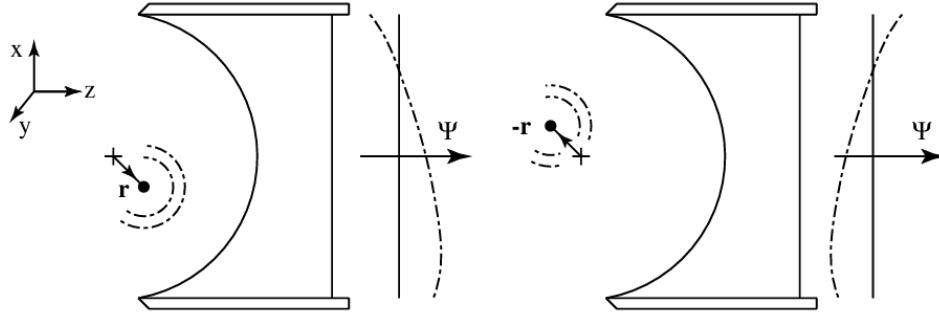


Figure 13. Schematic representing the odd parity of pupil function [13].

So, by optical reciprocity, if an opposing phase distribution is generated at a conjugate pupil plane, the aberrations introduced by the primary objective can be cancelled. Thus, when a secondary objective is placed in pupil conjugation and the magnification satisfies $\frac{\sin \alpha_1}{\sin \alpha_2} = \frac{\rho_2}{\rho_1}$, Consistent with Snell's law and Maxwell's condition, the phase distortions introduced by the first objective are compensated by those introduced by the second objective, so that a stigmatic replica of the object space is formed in the remote image space. In other words, the pupil phase generated by the first objective, Ψ_1 , is matched by an equal and opposite phase generated by the second objective, Ψ_2 , such that the two phase contributions cancel

$$\Psi_1(\rho_1, \phi) = kn_1 \sin \alpha \left(x\rho_1 \cos \phi + y\rho_1 \sin \phi + z \left(\frac{1}{\sin^2 \alpha_1} - \rho_1^2 \right)^{1/2} \right) \quad (17)$$

$$\Psi_2(\rho_2, \phi) = kn_2 \sin \alpha_2 \frac{n_1}{n_2} \left(x\rho_2 \cos \phi + y\rho_2 \sin \phi + z \left(\frac{1}{\sin^2 \alpha_2} - \rho_2^2 \right)^{1/2} \right) \quad (18)$$

A schematic of the complete remote-focusing setup is shown in the fig. 14,

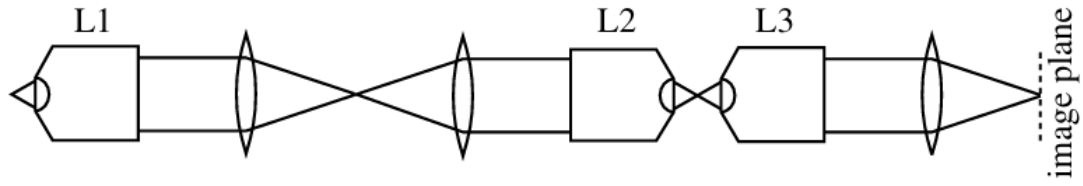


Figure 14. Schematic showing microscope with remote focusing [13].

This principle underlies conventional oblique plane microscopy (OPM), in which the obliquely illuminated plane of the specimen is first recreated in remote space through remote focusing relay optics and subsequently imaged by a tertiary objective at an oblique angle [12], [13],[28].

2.2 Dynamic remote focusing

In principle, aberration-free remote imaging can be achieved by compensating the additional wavefront phase introduced when a plane displaced from the focal plane is imaged in a high-magnification system designed according to the Abbe sine condition. When a point source is displaced axially, the resulting pupil phase contains a quadratic defocus term as well as higher-order contributions. If this additional phase can be properly compensated, the displaced plane may be brought into sharp focus without mechanical translation of the sample. Such phase compensation may be implemented using adaptive optical elements, including deformable mirrors, Alvarez lenses, deformable lenses, or electrically tunable lenses (ETLs) [29]. These devices introduce a controllable wavefront modulation at a plane conjugate to the objective pupil, thereby modifying the effective focal power of the system. However, unlike relay-based remote focusing employing a secondary objective that satisfies Maxwell's perfect imaging condition, adaptive phase compensation permits only one axial plane to be brought into focus at a given instant of time. In other words, the compensation is local in axial position and does not recreate a full three-dimensional replica of the object space instantaneously. Consequently, volumetric imaging using adaptive optical element like tunable-lens-based remote focusing requires dynamic modulation of the phase element. The focal power must be continuously varied to sequentially refocus different axial planes of

the sample. In the present microscope system, an ETL is employed for this purpose due to its simplicity, compactness, and cost-effectiveness. Modern ETL technology has matured significantly, providing stable focal power control over extended time periods and under varying environmental conditions. The ETL exhibits an approximately linear relationship between input current and focal power over its operating range [30]. By applying a ramped current waveform, the focal power can be smoothly varied from a minimum to a maximum value, thereby sequentially compensating for the defocus associated with different axial positions in the specimen [31]. The ETL is positioned at a plane conjugate to the pupil of the objective, ensuring that the introduced phase modulation corresponds to a controlled quadratic phase term at the pupil. This configuration enables rapid, inertia-free axial scanning without mechanical movement of the specimen [32]. Positioning the ETL at this location also helps maintain constant lateral magnification throughout the axial scan, which is important for preserving the correct image scale at different axial planes [33]. Using this technique, a new OPM system was realized for imaging along the oblique plane through scanning of both the illumination and detection. Fig. 15 illustrates a representative quadratic wavefront phase applied by the ETL at the pupil plane to compensate for defocus at a specific imaging depth. This quadratic phase is dynamically varied by the ETL, allowing the focal plane to be shifted while maintaining the image in focus.

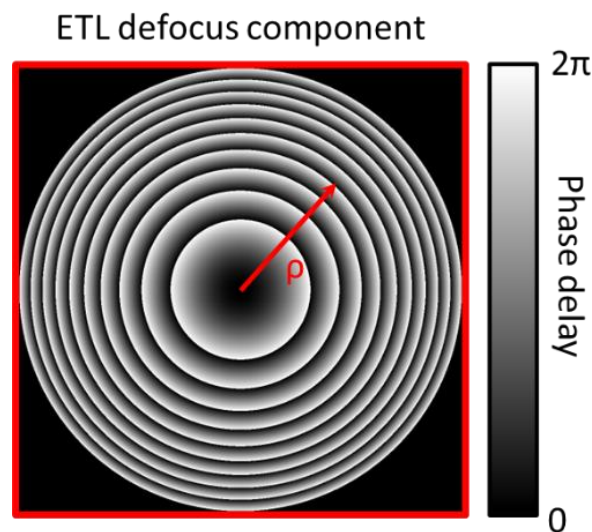


Figure 15. A representative defocus phase component introduced at the pupil plane by ETL at a specific imaging depth.

2.3 Optical Setup

In our full aperture oblique plane microscope, the excitation light sheet is generated using a cylindrical lens that focuses the laser beam along a single axis while maintaining collimation along the orthogonal axis [34]. This produces a thin sheet of light at the sample plane, enabling selective plane illumination. The excitation and emission paths are separated by a dichroic mirror, which reflects the excitation wavelengths toward the specimen while transmitting the longer-wavelength fluorescence emission into the detection arm. Appropriate emission filters further suppress residual excitation light to ensure high signal-to-background ratio.

Volumetric imaging is achieved through dynamic remote focusing. An ETL, positioned at a plane conjugate to the pupil of the primary objective, modulates the focal power of the system. Variation of the ETL introduces a controlled quadratic phase term at the pupil, thereby translating the oblique light sheet along its propagation direction within the sample. Because the ETL lies in a pupil-conjugate configuration, the emitted fluorescence undergoes reciprocal phase modulation. As a result, the illumination scan is accompanied by simultaneous optical de-scanning of the emission wavefront.

This dynamic phase compensation ensures that the illuminated oblique plane remains optically always conjugate to the detection focal plane. Consequently, the system is capable of imaging a tilted plane without employing a mechanically tilted detection geometry. Unlike conventional OPM implementations that rely on a physically tilted tertiary objective, the present configuration preserves a fixed detection plane while maintaining focus across the entire illuminated oblique sheet.

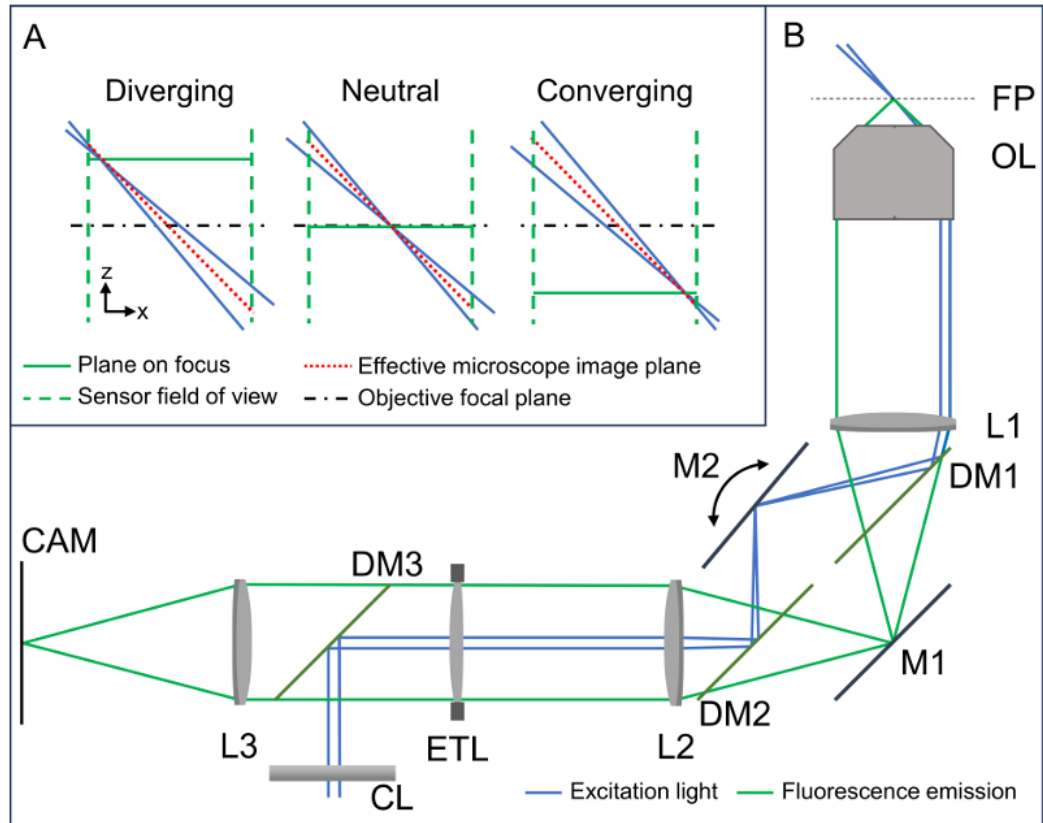


Figure 16. A) Scheme of the imaging geometry in the axial direction for different focal powers of the ETL. B) Simplified schematic of the optical setup of full aperture OPM with dynamic remote focusing [29].

Importantly, because the ETL-based remote focusing operates through pupil-plane phase modulation rather than tilted geometry, the full aperture of the primary objective is utilized for both illumination and detection. This allows the system to maintain the intrinsic resolution determined by the primary objective's numerical aperture, without sacrificing angular coverage due to tilted detection optics. A simplified schematic of the optical setup of full aperture OPM with dynamic remote focusing is given in fig. 16.

To further enhance optical sectioning, synchronized rolling shutter detection is employed. In rolling shutter mode, rows of pixels on the sCMOS sensor are exposed sequentially, forming a moving virtual slit. By synchronizing the motion of this virtual slit with the dynamic scanning of the oblique light sheet, only fluorescence from the in-focus region is recorded at any given instant. Out-of-focus emission is effectively rejected, producing confocal-like contrast while retaining the speed and reduced phototoxicity advantages of light-sheet illumination [35].

Through the combined action of cylindrical lens–based sheet formation, ETL-driven dynamic remote focusing, full-aperture utilization of the primary objective, and synchronized rolling shutter detection, the system achieves optically sectioned imaging of oblique planes without mechanical tilting or sample translation.

2.4 Comparison of full aperture OPM over conventional OPM systems.

While conventional oblique plane microscopy (OPM) relies on remote refocusing combined with a physically tilted tertiary objective to image an oblique plane, the present implementation achieves tilted-plane imaging through dynamic remote focusing without employing a mechanically tilted detection geometry. This architectural difference leads to several important distinctions in optical performance, system complexity, and flexibility.

In conventional OPM, the oblique re-imaging geometry often results in partial pupil clipping due to the angular acceptance limits of the tertiary objective. As discussed previously, this aperture mismatch can reduce the effective detection numerical aperture and introduce an anisotropic point spread function, which implies anisotropic resolution.

In contrast, the present system performs remote focusing via pupil-plane phase modulation using an ETL, without introducing a folded relay that truncates the pupil. As a result, the full aperture of the primary objective is preserved for both illumination and detection. Consequently, the intrinsic resolution of the objective is maintained without additional aperture-induced degradation.

Conventional OPM implementations typically employ a multi-objective relay system, including secondary and tertiary objectives arranged in a tilted configuration. The present system, by comparison, operates effectively as a single-objective configuration for both illumination and detection, with dynamic phase modulation replacing geometric re-imaging. This reduces optical complexity and eliminates the need for precise angular alignment between multiple high-NA objectives making it much less complex to align and maintain. The absence of mechanically tilted

detection optics significantly simplifies alignment. In conventional OPM, careful adjustment of the tilted tertiary objective is required to ensure accurate conjugation of the oblique plane. Small misalignments can introduce aberrations or spatial distortions. In the present system, the detection plane remains fixed and orthogonal to the optical axis, while the oblique plane is maintained in focus through dynamic remote focusing. This reduces alignment sensitivity and improves mechanical stability. This configuration also preserves the telecentricity of the system, unlike some conventional OPM.

By avoiding pupil clipping and tilted relay constraints, the present configuration provides greater flexibility in objective selection. Objectives with different numerical apertures can be employed, provided that the pupil is properly matched to the ETL and relay optics. In principle, this architecture allows switching between objectives using a turret mechanism, enabling adaptation to different imaging requirements such as high-resolution or large field-of-view operation.

While conventional OPM systems are often optimized for high NA operation to preserve angular coverage in the tilted relay geometry, many biological imaging applications require lower NA objectives to achieve a larger FoV. Mesoscopic imaging, developmental biology studies, and large tissue mapping frequently prioritize spatial coverage over diffraction-limited resolution. In such cases, the use of lower-NA objectives is advantageous due to their inherently larger FoV and longer working distance.

In conventional OPM architectures, the requirement for angular overlap between the primary, secondary, and tertiary objectives can restrict the use of low-NA objectives, as reduced angular coverage may exacerbate pupil truncation and compromise tilted-plane imaging. As a result, system design may be constrained toward high-NA objectives even when the application demands larger imaging volumes.

In contrast, the present system employs dynamic remote focusing through pupil-plane phase modulation rather than geometric re-imaging with tilted objectives. Because the full pupil of the primary objective is preserved and no angular relay clipping is introduced by a tilted tertiary objective, the system architecture does not

inherently impose a high-NA requirement. This enables greater flexibility in selecting objectives based on application needs.

For applications requiring high spatial resolution, high-NA objectives can be employed to exploit the full aperture of the system. Conversely, for large-area or mesoscopic imaging, lower-NA objectives can be used to expand the accessible FoV without being constrained by relay aperture matching conditions. In principle, provided that the pupil is properly matched to the ETL and relay optics, objective switching via a turret mechanism is feasible, allowing rapid reconfiguration of the microscope for different imaging regimes which makes it unique among other OPM system.

2.5 Simulation framework for full-aperture OPM

A simulation framework based on the angular spectrum method discussed earlier was used to model the effective three-dimensional response of the full-aperture OPM system. Although the detection aperture is preserved in this architecture, the final imaging response depends on the coordinated action of oblique light-sheet illumination, ETL-based dynamic remote focusing, galvo scanning, rolling-shutter detection, and deskewed volume reconstruction. The purpose of the simulation was therefore to evaluate how these processes combine to determine the effective PSF and resolution of the system.

The previous section described the operating principle of the full-aperture OPM system developed in this work. Unlike conventional OPM architectures, in which the oblique image plane is physically re-imaged onto the camera using a tilted tertiary detection path, the present system preserves the detection aperture and does not rotate the detection plane to match the oblique illumination plane. Instead, dynamic remote focusing is used to scan the detection focus, while synchronised rolling-shutter detection provides optical sectioning by acting as a virtual confocal slit. A numerical simulation framework was therefore developed to analyse how these coordinated illumination, detection, remote-focusing, and rolling-shutter processes determine the effective system PSF.

The aim of the simulation was not to reproduce the conventional tilted-tertiary OPM geometry considered in Chapter 1, but to model the image-formation mechanism of the full-aperture dynamic remote-focusing system. In this configuration, the illumination light sheet remains oblique in the y - z plane, while the detection focal plane remains the x - y plane. The simulation therefore focuses on how the scanned illumination, dynamically shifted detection focus, and rolling-shutter exposure window combine to produce optical sectioning and determine the final three-dimensional response.

This simulation therefore differs from the conventional OPM model discussed in Chapter 1. No tilted tertiary-objective detection arm or frequency-domain rotation of the detection pupil is used. Instead, the model focuses on the coordinated action of the light-sheet illumination, dynamic defocus sweep, detection PSF, rolling-shutter gating, and final deskewing step. The resulting simulated PSF is used to evaluate how these processes combine to determine the resolution and optical-sectioning behaviour of the full-aperture OPM system.

2.5.1 Coordinate system and simulation geometry

The coordinate system used in the simulation is defined with respect to the detection path. The detection optical axis is taken as z , and the nominal detection focal plane is the x - y plane. The oblique illumination plane is tilted in the y - z plane, while x remains the transverse direction perpendicular to the tilt plane. This convention is used consistently for the illumination PSF, detection PSF, rolling-shutter model, and final deskewed reconstruction.

This coordinate definition is particularly important for the full-aperture dynamic remote-focusing system because the detection plane is not physically rotated to match the illumination plane. Instead, axial refocusing is produced dynamically, and optical sectioning is recovered through synchronisation between the scanned illumination and the rolling-shutter exposure window. The simulation therefore keeps the detection coordinate system fixed and models the apparent oblique acquisition geometry through the illumination position, defocus sweep, rolling-shutter gating, and deskewing transformation.

2.5.2 Angular-spectrum propagation and dynamic defocus modelling

The same angular-spectrum propagation framework was used to calculate both the illumination and detection responses from their corresponding pupil functions. The detection path was represented by a centred circular pupil defined by the detection numerical aperture. The illumination path was represented by a shifted slit pupil, similar to the light-sheet pupil used in Chapter 1, corresponding to the off-axis illumination aperture produced by the illumination scan mirror.

Since both the illumination and detection beams pass through the electrically tunable lens, the defocus phase was applied to both paths. However, the defocus term was treated differently for the two pupils. For detection, the defocus phase was centred on the optical axis, whereas for illumination it was centred on the shifted slit pupil. This shifted defocus treatment accounts for the fact that the ETL-induced phase curvature acts on an off-axis illumination beam rather than on a centred pupil.

For each ETL defocus position, the illumination and detection fields were propagated through a set of axial planes to generate separate three-dimensional PSF stacks. These stacks were then used to model the synchronized rolling-shutter acquisition, where only the rows corresponding to the illuminated and exposed region contribute to the accumulated signal.

Before applying the rolling-shutter model, the simulated response was interpreted as the PSF of an ideal fluorescent point emitter. This follows the same light-sheet image-formation principle introduced in Chapter 1, where the effective fluorescence response depends on both illumination and detection. In the present point-emitter simulation, the illumination intensity at the bead position was used as a scalar excitation weight, while the spatial distribution of the emitted fluorescence was described by the corresponding detection PSF. The bead response for each ETL defocus state was therefore calculated from the illumination-weighted detection PSF. This representation isolates the optical response of the system and does not include convolution with a finite bead diameter.

The galvo scan was then introduced to represent the lateral sweep of the light sheet across the point emitter. For each scan position, the illumination intensity at the bead location changes according to the overlap between the shifted light sheet and the bead. In the simulation, this produces a brightness envelope, in which the signal rises as the light sheet reaches the bead and decreases as it moves away. This step represents the scanned illumination component of the acquisition and provides the illumination weighting used in the subsequent rolling-shutter model.

The rolling-shutter detection process was then introduced by defining an exposed strip around the position of the illumination maximum for each ETL defocus step. This strip represents the camera rows that are active during the corresponding part of the acquisition and acts as a virtual confocal slit when synchronized with the scanned light sheet. Only the detection signal within this exposed strip was accumulated, producing the post-rolling-shutter bead PSF.

The post-rolling-shutter bead PSF represents the signal recorded in the camera frame during the synchronized acquisition. Because the light sheet is scanned obliquely, the raw image stack is geometrically skewed with respect to the sample coordinate system. A deskewing step is therefore required to reconstruct the final three-dimensional PSF in sample-oriented coordinates. In the simulation, this reconstruction was performed using an affine transformation, which is described in detail in the following section.

After deskewing, the reconstructed volume was interpreted in the sample-oriented coordinate system, with the reconstructed array axes ordered as z , y , and x . The final reconstructed bead PSF was normalised to its maximum intensity and analysed by extracting one-dimensional intensity profiles through the PSF peak along each axis. The full width at half maximum was then calculated from these profiles to estimate the effective axial and lateral resolution of the simulated system.

This analysis provides a quantitative measure of the combined effect of illumination, detection, ETL-based dynamic refocusing, rolling-shutter gating, and deskewed reconstruction on the final system response. The resulting FWHM values therefore describe the effective resolution of the full-aperture OPM simulation rather than the detection PSF alone.

The numerical values used for the simulation, including the wavelength, numerical aperture, grid size, defocus range, axial sampling, galvo scan range, and rolling-shutter width, were kept fixed throughout.

The reconstructed bead PSF obtained from the full-aperture OPM simulation is shown in fig. 17. The PSF represents the final system response after illumination weighting, detection propagation, ETL-based dynamic refocusing, galvo scanning, rolling-shutter gating, and affine deskewing. Therefore, the displayed PSF corresponds to the effective reconstructed response of the complete simulated acquisition process, rather than to the illumination or detection PSF alone.

The reconstructed PSF was analysed along the z, y, and x directions by extracting intensity profiles through the maximum-intensity voxel. The corresponding FWHM values were used to quantify the effective axial and lateral resolution of the simulated system. The measured widths were approximately 4.2 μm along z, 570 nm along y, and 560 nm along x.

The lateral FWHM values are close to the diffraction-limited detection reference value of 532 nm for wavelength divided by twice the numerical aperture of 0.5, indicating that the simulated reconstruction preserves lateral resolution in the camera-plane directions. In contrast, the axial response is broader and is governed by the combined effect of the finite light-sheet thickness, rolling-shutter gating, and reconstruction geometry. Thus, the axial FWHM should be interpreted as the effective sectioning response of the full simulated acquisition process, rather than as the axial detection PSF alone.

The axial FWHM was approximately 4.2 μm , which is broader than the axial detection reference of 2.13 μm estimated from the wavelength divided by the square of the numerical aperture. This broadening is expected because the axial response in the reconstructed OPM volume is not determined by the detection PSF alone. Instead, it reflects the combined effect of the finite light-sheet thickness, rolling-shutter gating, ETL-based refocusing, and affine deskewing. The axial FWHM therefore represents the effective sectioning response of the simulated acquisition process.

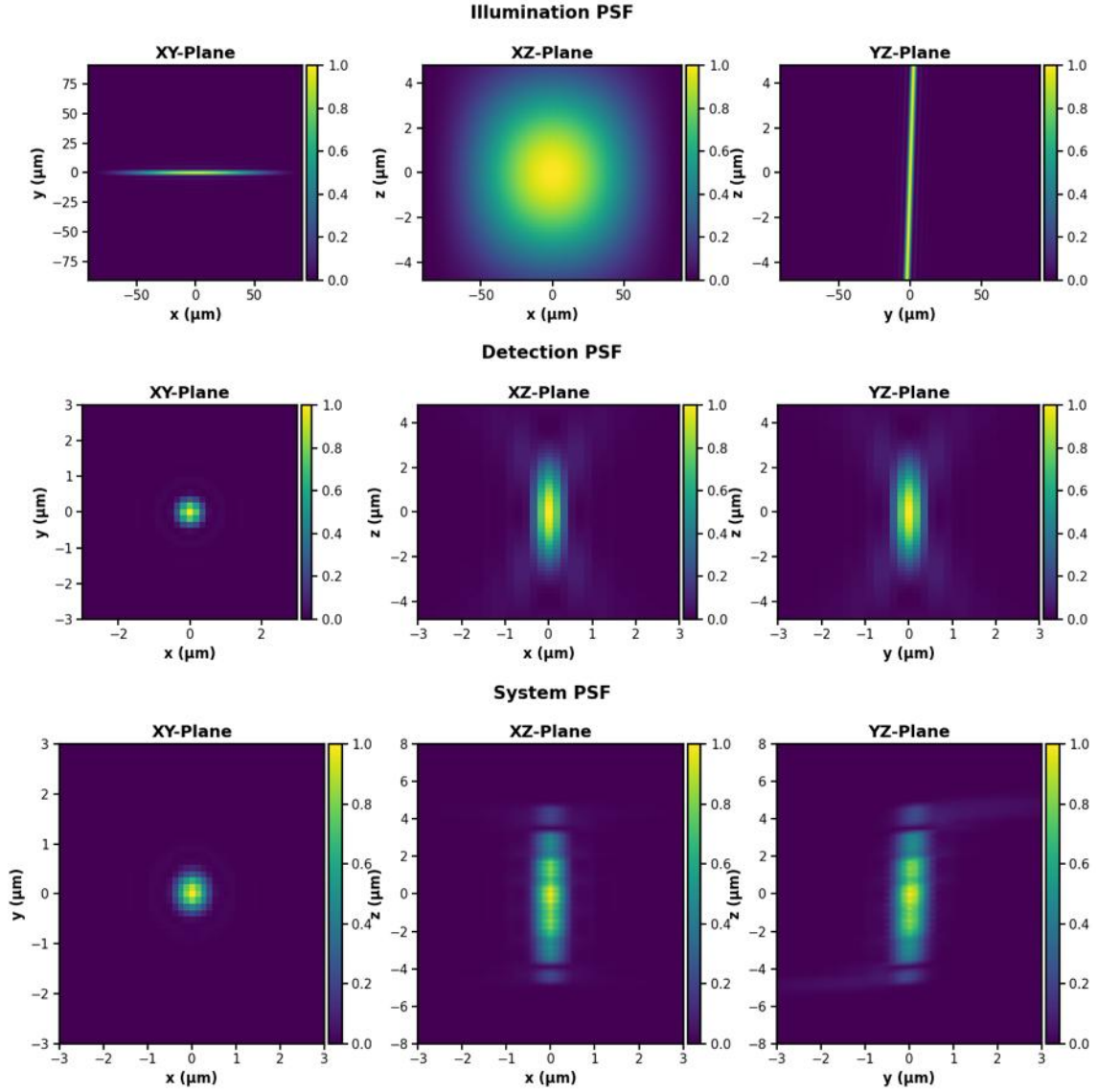


Figure 17. Reconstructed bead PSF obtained from the full-aperture OPM simulation after illumination weighting, detection propagation, dynamic refocusing, galvo scanning, rolling-shutter gating, and affine deskewing. Orthogonal views of the reconstructed PSF are shown in the x - y , x - z , and y - z planes.

Overall, the simulations provide a diffraction-based estimate of the effective reconstructed response of the full-aperture OPM system. The lateral FWHM values remain close to the detection-limited reference, while the broader axial FWHM reflects the combined influence of finite light-sheet thickness, rolling-shutter gating, ETL-based refocusing, and deskewed reconstruction. These results provide a basis for interpreting the expected resolution of the system and for comparison with experimentally measured PSFs.

3. System implementation and validation

3.1 Overview of the optical system.

The current optical setup consists of two laser sources; during the initial phase of microscope development, a laser at 473 nm (Lambda beam, RGB Photonics) and another at 561 nm (06-DPL, Cobolt) were used. Later, it was changed to a single multi-wavelength laser source with 488 nm and 561 nm (CellX, Coherent). The two lasers were fiber-coupled to two reflective-type beam expanders, which collimated and expanded the beam to the desired dimensions. These two expanded laser beams are perfectly aligned to the optical axis with the mirror and the dichroic. The coherent laser used in the latter stage is a fiber pigtailed laser, which was collimated and expanded to the desired beam size using a 30 mm focal lens. After this stage, the combined two-colour beam is passed through a cylindrical lens of 400 mm focal length to form the light sheet. Then this light sheet is focused onto the galvo with a 150 mm focal length lens. The reflection from the galvanometric mirror is coupled to the ETL (EL-16-40-TC, Optotune) through a telescope formed by lenses of focal length 180 mm and 400 mm, respectively (magnified by 2.2x). The ETL is placed at a plane conjugate to the back focal plane of the objective lens. Light sheet is then coupled to the back focal plane of the objective through a telescope formed by lenses of 400 mm and 200 mm focal length (0.5x). The illumination and detection path are split using multiband dichroic mirrors located in between this telescope. Then the light sheet is focused onto the sample by the objective. Two objectives Olympus LUMFL 60x1.1N.A and Olympus (UMPLFN) and 20x 0.5 N.A water immersion objectives were used for the experiment which were switched in between during the experiment using a turret. Samples were mounted on an ASI MS-2000-500-UDSW micrometre translational stage. The fluorescent emission from the sample is relayed back through the same telescope formed by 200 mm and 400 mm lens with 2x

magnification which matches the full aperture of the ETL (16 mm, pupil 6.6 mm for 60x). The emission is relayed from ETL to galvo through 400 mm and 180 mm telescope, this is focused on the camera using 200 mm tube lens through Hamamatsu W-view Gemini wavelength splitter which splits the two-colour fluorescent emission. The camera is Hamamatsu orca flash 4.0. The sensor area is split into half for two colours. ETL and galvo are synchronously controlled by a National Instrument DAQ which is run by a custom PyQt based software. A simplified schematic of the current setup is shown in fig. 18.

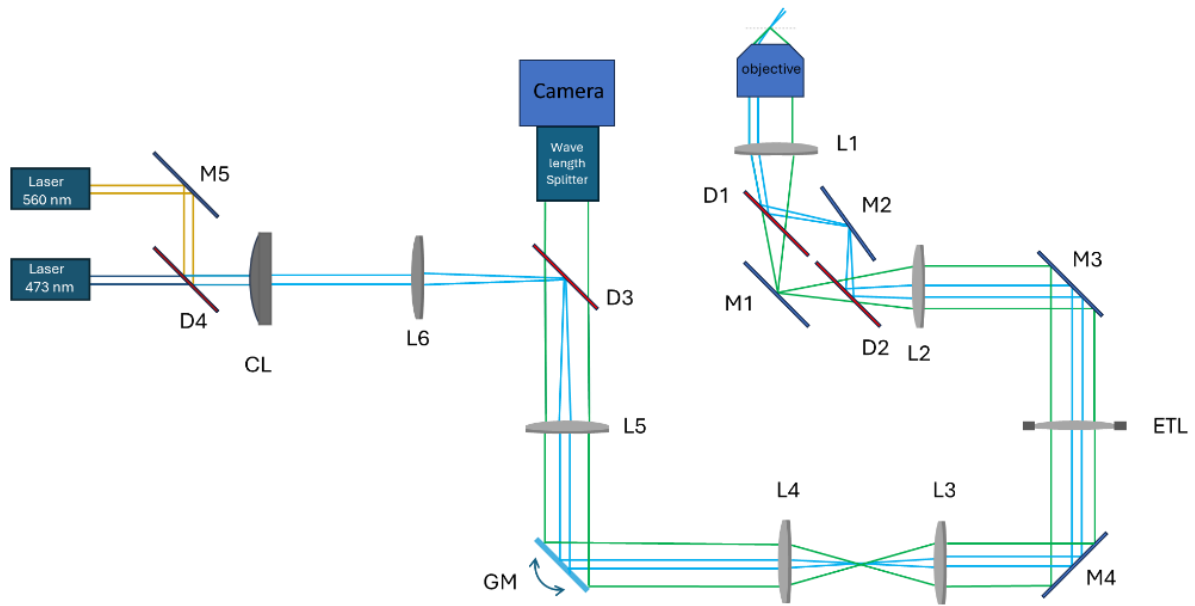


Figure 18. Schematic representing full aperture OPM with dynamic remote focusing with galvo mirror (GM) scanning and dual colour capability.

3.2 Electrically Tunable Lens

The electrically tunable lens (ETL) constitutes a central component of the present imaging system. The ETL used in this work (EL-16-40-TC, Optotune)(fig.19a) is a focus-tunable lens based on shape-changing polymer membrane technology. By applying an electrical current, the curvature of the lens is dynamically adjusted, enabling rapid modulation of focal power within millisecond timescales (fig. 19b). The device provides a focal power tuning range from -10 dioptres (concave configuration) to $+10$ dioptres (convex configuration).

The lens consists of an optical fluid sealed within a transparent chamber by an elastic polymer membrane. An electromagnetic actuator based on voice-coil technology exerts pressure on the fluid-filled chamber, thereby deforming the membrane and altering the curvature of the optical interface. The focal power of the lens is therefore directly controlled by the current applied to the voice coil. Because the deformation is continuous and reversible, smooth axial scanning can be achieved without mechanical translation of bulk optical components.

The dynamic response of the ETL is governed by the mechanical properties of the membrane–fluid system. The device exhibits a mechanical resonance frequency of approximately 400 Hz. While this resonance defines the upper limit of the system’s mechanical response, stable and distortion-free operation typically requires driving frequencies well below resonance, depending on the modulation amplitude. For small-amplitude focal power modulation, the lens can operate at tens to hundreds of hertz. Larger scan amplitudes require lower operating frequencies to avoid oscillatory behaviour and phase lag [29], [32].

To ensure repeatability and long-term stability, the lens incorporates an integrated temperature sensor that enables compensation for thermally induced changes in focal power. This is particularly important in dynamic scanning applications where resistive heating of the actuator may otherwise introduce drift.

The manufacturer specifies a wavefront error of approximately 0.15λ RMS for class 1 devices measured under standard conditions. When the lens is oriented with its optical axis horizontal (i.e., standing upright), gravitational effects can introduce an additional Y-coma component due to asymmetric deformation of the membrane. In this configuration, the total wavefront error may increase to approximately 0.5λ RMS. The magnitude of this gravity-induced aberration depends on factors including the clear aperture of the lens, the density of the optical fluid, and the mechanical properties of the polymer membrane.

In the present system, the ETL is positioned at a plane conjugate to the objective pupil placed horizontally, where it introduces a controlled quadratic phase modulation corresponding to defocus. This configuration enables rapid, inertia-free axial scanning while minimizing mechanical perturbation of the sample [30].

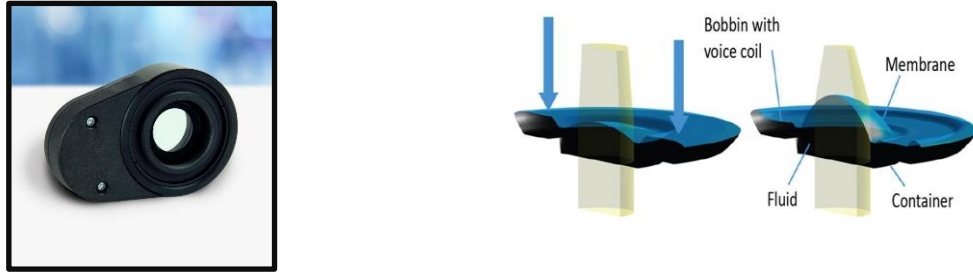


Figure 19. a) Optotune electrically tunable lens. b) Focal power changed by changing the curvature of the membrane by the voice coil [30].

3.3 Synchronisation

Three-dimensional imaging requires optically sectioned images to ensure that only in-focus fluorescence contributes to the reconstructed volume. In conventional confocal microscopy, optical sectioning is achieved by spatial filtering using a pinhole that rejects out-of-focus emission [36]. In light-sheet fluorescence microscopy (LSFM), optical sectioning is instead achieved through selective plane illumination: only fluorophores within the focal plane of the detection objective are excited, inherently suppressing background signal from other axial positions. In conventional LSFM, the illumination plane coincides with the x - y detection focal plane [8].

In oblique light-sheet microscopy, however, the illumination plane intersects the x - y focal plane at an angle rather than coinciding with it. As a result, the excited region is no longer confined to a single axial position z , and fluorescence originates from a tilted plane with both lateral and axial extent, rather than from the x - y focal plane alone. In traditional oblique plane microscopy (OPM), this issue is addressed by remote refocusing in combination with tilted detection optics, which physically reorient the image plane so that the entire obliquely illuminated plane is brought into focus on the camera. In this configuration, optical sectioning is preserved because the obliquely illuminated plane is optically reoriented into a plane perpendicular to the detection axis, thereby restoring the geometry of conventional light-sheet microscopy.

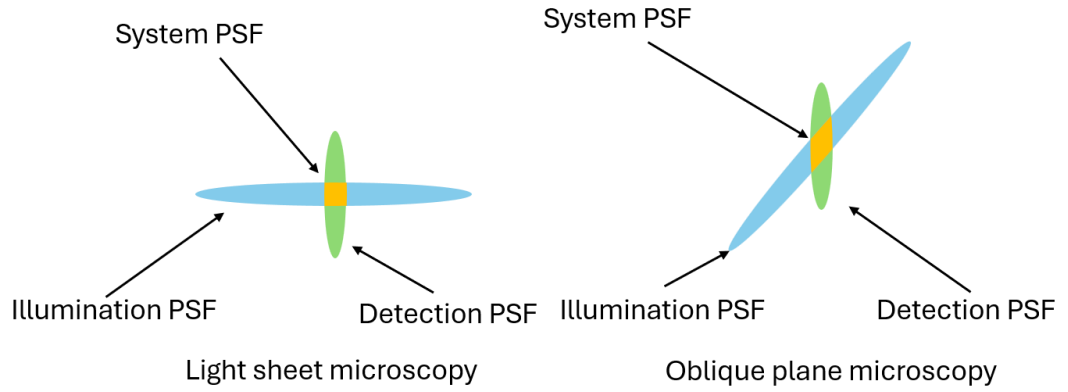


Figure 20. Schematic representing the axial resolution of a conventional light-sheet microscope and the axial resolution of a full-aperture OPM with dynamic remote focusing, and the illumination and detection PSF (Adapted from [37]).

In contrast, the present system employs an oblique scanning configuration without rotating the detection plane to coincide with the illumination plane. As a result, at each instant of the oblique light-sheet scan, fluorescence is excited not only within the lateral detection focal plane but also in regions with a finite axial offset. Consequently, part of the detected fluorescence originates from planes that are defocused with respect to the detection focal plane. This out-of-focus fluorescence reduces image contrast and compromises the optical sectioning capability that is otherwise intrinsic to LSFM and OPM. The extent of defocus that will be present in the recorded image is governed by the overlap between the detection depth of focus and the Rayleigh range of the illumination beam as shown in the fig. 20. To restore optical sectioning under these conditions, out-of-focus fluorescence must be rejected either physically or computationally. A direct physical approach involves placing a slit aperture in the emission path at a plane optically conjugate to the camera plane [38], [39]. However, implementations in which the slit remains fixed, and the emitted fluorescence is scanned across it using a galvanometric mirror introduce additional system complexity. An alternative and more efficient method is to use the rolling shutter mode of an sCMOS camera as a virtual confocal slit. Modern sCMOS cameras operate in either global shutter or rolling shutter modes. In global shutter mode, all pixels are exposed simultaneously and read out together. In many modern sCMOS cameras, the sensor readout is inherently rolling shutter; however, a global-exposure interval can be generated during which all sensor rows are exposed simultaneously, thereby approximating global-shutter acquisition [40][41]. In rolling

shutter mode, rows of pixels are exposed sequentially with a defined line interval and exposure duration. At any given time, only a subset of adjacent pixel rows is active, effectively forming a moving slit that traverses the sensor. This rolling exposure window therefore acts as a dynamic slit without requiring additional mechanical components. When its motion is synchronized with the scanned light-sheet illumination, it becomes a virtual confocal slit such that only in-focus fluorescence is detected while out-of-focus emission is rejected. This significantly improves image contrast and signal-to-noise ratio (SNR), restoring the optical sectioning capability of the system [42]. The Hamamatsu sCMOS camera used in this work offers multiple rolling-shutter readout configurations. For the present system, Light-Sheet Readout Mode is the most appropriate, since it performs unidirectional sensor readout from top to bottom, thereby simplifying synchronization with the scanned light sheet as shown in the fig. 21.

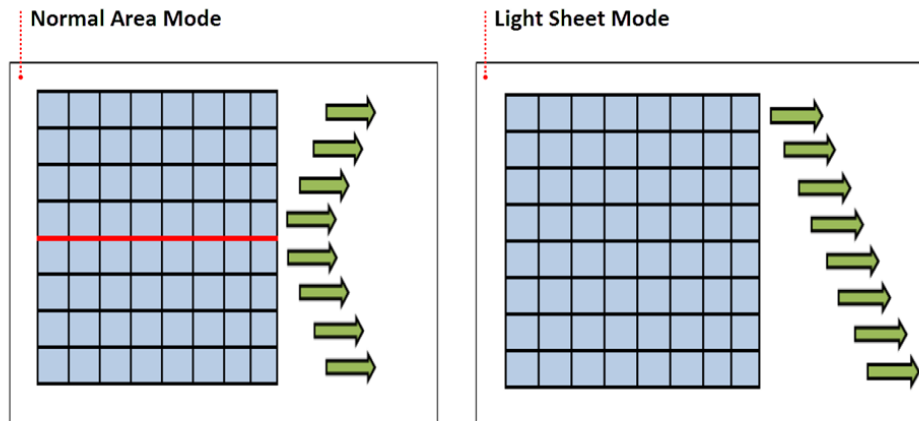


Figure 21. Readout modes available in the sCMOS camera: Normal Area Mode and Light Sheet Mode. In Normal Area Mode, readout starts from the central sensor line and proceeds simultaneously towards the top and bottom of the sensor. In Light Sheet Mode, the sensor is read sequentially in a single direction, either from top to bottom or from bottom to top [43].

Proper synchronisation requires careful consideration of both illumination and camera parameters. The scanning frequency of the light sheet is determined by the operating frequency of the ETL, which controls axial defocus and thereby the movement of the illumination across the sample. The spatial extent of the scan on the camera sensor is determined by the optical power range of the ETL, which in turn depends on the amplitude of the driving current. On the detection side, key camera parameters include the line readout speed which determines the rolling shutter

velocity, the exposure time which determines the effective slit width, and the maximum achievable readout rate set by the camera electronics. The exposure time directly controls the thickness of the virtual confocal slit and thus influences the degree of background rejection. For effective optical sectioning, the rolling shutter motion must be aligned with the scanning illumination. This requires synchronous triggering of both the camera and the ETL. Although both devices can be triggered simultaneously, electronic latency within the camera, ETL driver, and signal routing introduces timing offsets. These delays are compensated for by adjusting the phase delay of the sawtooth waveform driving the ETL, ensuring that the illuminated region of the sample coincides precisely with the active rolling shutter window.

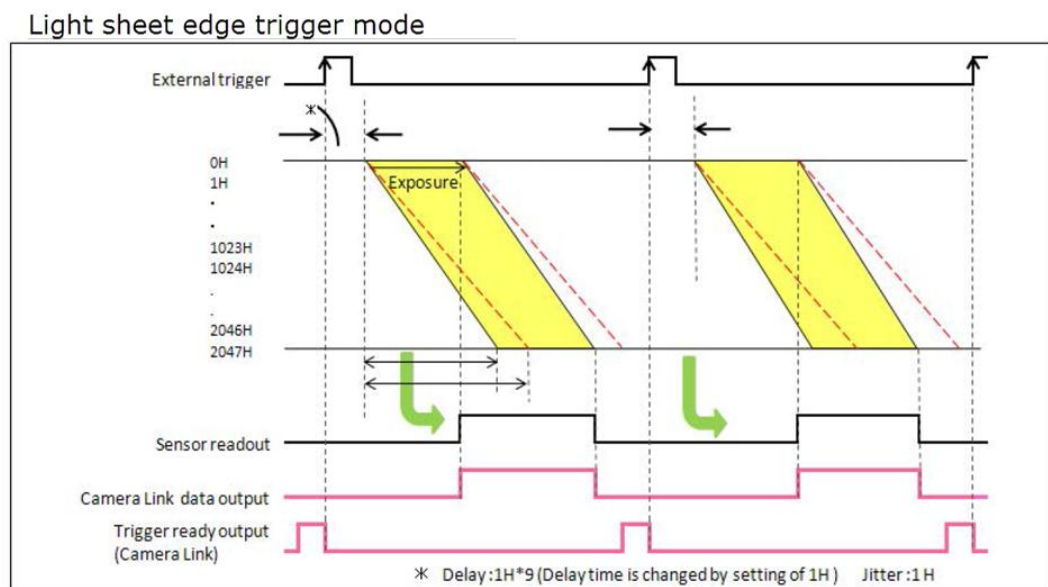


Figure 22. Timing diagram of the light sheet mode in the Hamamatsu ORCA Flash sCMOS camera used for synchronized rolling shutter. An external trigger initiates image acquisition by the camera, providing synchronisation between the camera readout and the light-sheet scanning [43].

When the camera receives an external trigger from the National Instruments digital output in edge-trigger mode, exposure of the first pixel line begins, as shown in the timing diagram in fig. 22. Each subsequent line is then exposed after a fixed line delay. Once the specified exposure time has elapsed, sensor readout begins and proceeds sequentially on a line-by-line basis. The line delay determines the slope of the rolling exposure window in the timing diagram and therefore reflects the speed at which the rolling shutter propagates across the sensor. The exposure time determines when readout begins for each line and, together with the line timing, sets

the total frame duration and hence the achievable frame rate. The readout data are transmitted through the camera link interface simultaneously with sensor readout. Once both readout and data transmission are complete, the camera outputs a trigger-ready signal. These two parameters exposure time and line delay are primarily used to achieve synchronization between the rolling shutter and the scanned light-sheet illumination [41].

3.4 Procedure for synchronization

Synchronization is achieved when the rolling-shutter detection window traverses the sensor at the same effective speed as the scanned light sheet traverses the sample. Once synchronization is achieved, these two movements become coupled and effectively propagate together. However, if they are treated separately and make these movements as mutually perpendicular movement, their intersection follows a perfect diagonal across the square sensor area (fig. 23). This concept is used as the synchronization procedure. First light sheet is rotated by 90 degrees by rotating the cylindrical lens, and the oblique angle of illumination is changed from scanning parallel to the rolling shutter to perpendicular to the rolling shutter direction. Or just rotating the camera by 90 degree can give the same effect. This mode is referred to as synchronisation mode apart from normal imaging mode as shown in the fig. 23. A uniform fluorescent slide is used as the sample. The light sheet is scanned perpendicular to camera sensor area and rolling shutter sample the lightsheet to form the image.

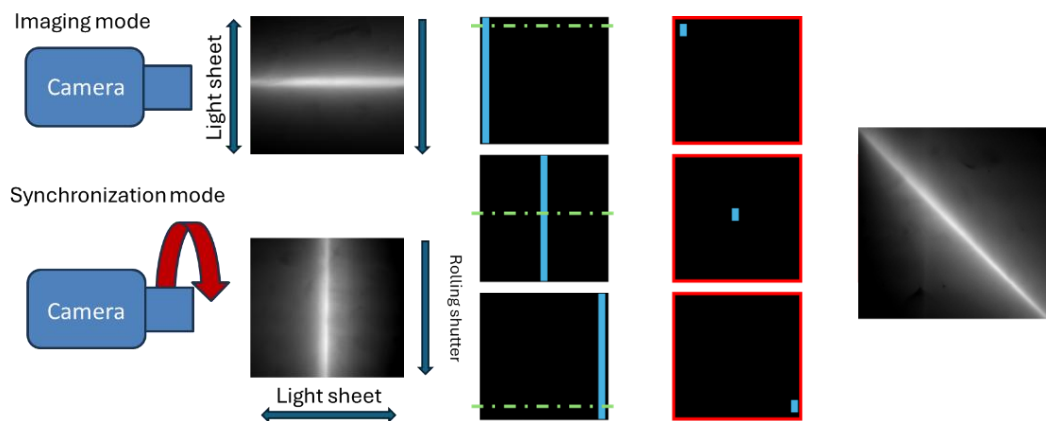


Figure 23. Image showing the imaging mode and synchronisation mode for image acquisition with schematics of sampling of light sheet by rolling shutter in synchronisation mode.

The exposure time, line speed, and phase delay of the sawtooth waveform driving the ETL are adjusted to obtain a perfect diagonal formed by the light sheet across the sensor area. The effect of each parameter on synchronisation is illustrated in the fig. 24. The exposure time primarily determines the width of the confocal slit, whereas the line speed and phase delay determine the position of the light sheet within the field of view.

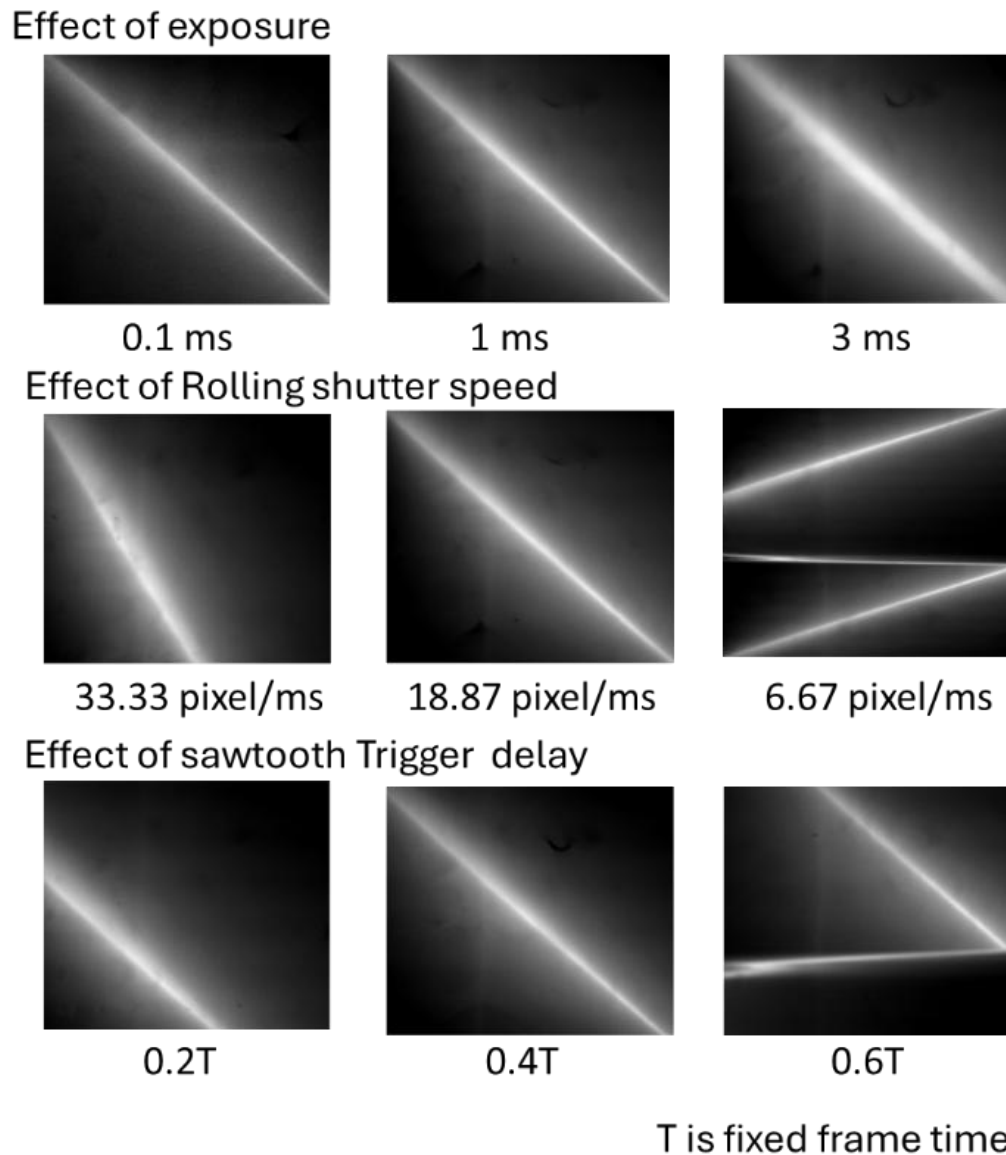


Figure 24. Image showing the effects of camera exposure time, line speed, and the phase delay of the driving sawtooth waveform on the synchronisation between the light sheet and the rolling shutter.

Once synchronization has been achieved, the light sheet is returned to its initial position corresponding to imaging mode. The system can then be operated in imaging mode to acquire an optically sectioned image. So, synchronisation brings

back the optical sectioning, and the oblique plane can be imaged perfectly without defocus.

3.5 Mechanical scanning and galvo scanning

The maximum FoV achievable in a fluorescence microscope is fundamentally limited by the region of the sample that is illuminated and imaged with sufficient optical quality. In widefield microscopy, the entire FoV of the objective is illuminated simultaneously, allowing full utilization of the objective's imaging area within the focal plane. As a result, the entire accessible FoV can be recorded in a single acquisition.

In contrast, advanced microscopy techniques such as confocal microscopy and oblique light-sheet microscopy illuminate only a subset of the objective's maximum FoV at any given time. Consequently, imaging larger sample areas requires an additional scanning mechanism to extend the effective imaging region beyond the instantaneous illuminated area to achieve full FoV of the objective [44] .

Two primary scanning approaches are commonly employed: optical scanning and mechanical scanning. In optical scanning, the illumination beam is deflected using galvanometric mirrors positioned at the back focal plane (BFP) of the objective or at a plane conjugate to the BFP. Angular deflection at the pupil plane translates into lateral displacement at the sample plane. Because small angular changes correspond to large spatial shifts in sample space, galvanometric scanning enables rapid beam movement with high precision [45], [46].

Optical scanning offers several advantages:

- High scanning speed
- Minimal mechanical inertia
- No physical perturbation of the sample
- Reduced motion artifacts

However, the maximum scanning range achievable by galvanometric deflection is inherently limited by the objective's FoV. For high-NA objectives, this FoV is typically

on the order of a few hundred micrometres, which may be insufficient for imaging large biological specimens whose dimensions can extend to millimetre scales.

In mechanical scanning involves physically translating the sample relative to the illumination. This is typically achieved using multi-axis translation stages, including motorized stages or piezo-driven actuators. Mechanical stages provide a significantly larger scan range and, in principle, allow imaging over arbitrarily large areas.

The primary limitation of mechanical scanning arises from inertia. Because the stage and sample possess mass, rapid accelerations and decelerations are constrained by mechanical limits. This restricts achievable scanning speed, particularly in applications requiring repeated imaging of the same region at short time intervals. Additionally, mechanical motion can introduce vibrations or agitation, potentially perturbing delicate biological samples and generating motion artifacts [47]. Images of the galvanometric scanner and mechanical translation stage used in the setup are shown in fig. 25.



Figure 25. A Thorlabs galvanometric scanner [48] and an ASI MS-2000 multi-axis translation stage [49].

3.6 Dual colour imaging

Simultaneous imaging of multiple structures within a biological sample is essential for studying spatial organization and dynamic interactions. This is typically achieved by labelling distinct structures with fluorophores that exhibit well-defined and spectrally separated excitation and emission wavelengths. Accurate multicolour imaging requires that the optical system preserve spatial correspondence across channels, ensuring that images from different fluorophores are geometrically co-registered.

To achieve this, the entire optical system was designed to minimize chromatic aberration. If chromatic effects are not properly controlled, wavelength-dependent focal shifts and angular deviations can occur. In excitation, different wavelengths may focus on slightly different axial positions or propagate at different angles through the illumination optics. In an oblique illumination configuration, this can lead to wavelength-dependent variation in the illumination angle, focal position, and beam alignment. Such deviations can result in channel-dependent differences in magnification, resolution, and illumination angle.

In detection, chromatic aberration can introduce lateral and axial image shifts between channels. If uncorrected, this may cause misregistration between colour channels, resulting in inaccurate spatial overlap. In systems employing synchronized rolling shutter detection, chromatic focal shifts can additionally disrupt the temporal alignment between the scanned illumination and the virtual confocal slit, thereby degrading optical sectioning and reducing image resolution.



Figure 26. W-View Gemini wavelength splitter used for two-color imaging [50].

To mitigate these effects, all relay optics were selected as achromatic doublets corrected over the visible spectral range. The primary objective used in this system is an apochromatic objective, providing enhanced correction for chromatic aberration across multiple wavelengths. Multiband dichroic mirrors and emission filters were employed to ensure efficient wavelength separation while maintaining minimal angular deviation across channels [2], [50].

In the detection path, a Hamamatsu W-View Gemini wavelength splitter is used, with optics designed to provide adjustable magnification correction (fig. 26). A two-colour calibration target provided with wavelength splitter was used to quantify residual magnification mismatch and spatial offsets between channels, allowing post-alignment correction where necessary. Through careful optical alignment, channel-dependent aberrations were minimized, ensuring accurate multicolour co-registration and preservation of optical sectioning performance. The camera was operated in dual-colour rolling shutter mode to simultaneously acquire the two separated colour channels (fig. 27).

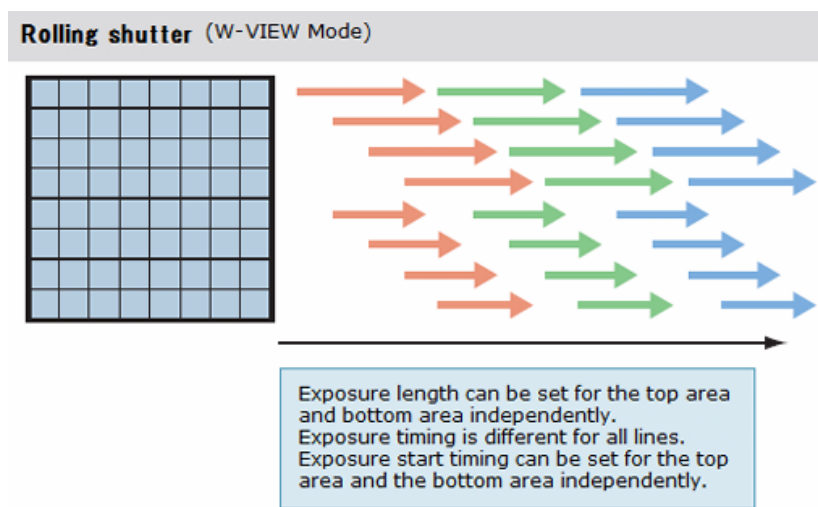


Figure 27. The dual rolling-shutter mode of the sCMOS camera enables simultaneous readout of the two channels [43].

3.7 Reshaping of data set

In conventional light-sheet fluorescence microscopy, the illumination plane is orthogonal to the detection focal plane, and volumetric imaging is achieved by translating the sample through the light sheet. Each acquired image corresponds directly to a true axial slice of the specimen [32].

In oblique light-sheet microscopy, the illumination plane is inclined relative to the detection focal plane. In traditional OPM, this geometric mismatch is resolved optically: the illuminated oblique plane is re-imaged through remote refocusing and a tilted tertiary objective such that the camera directly records a true oblique slice of the specimen. Volumetric imaging is then achieved by scanning the light sheet and

acquiring successive oblique slices. Because the imaging plane itself is tilted, each recorded frame represents a geometrically accurate section of the sample. The resulting stack of oblique slices can be computationally transformed into a conventional cartesian volume by applying an affine transformation consisting of scaling and rotation [51], [52].

In the present system, however, the detection plane is not rotated to match the oblique illumination plane. Instead, the camera records a projection of the oblique illuminated plane onto the fixed detection focal plane. Consequently, each acquired image does not represent a true oblique section but rather a geometrically distorted projection of that section. This projection introduces a scaling factor along the axis of obliqueness due to the angular relationship between the illumination plane and the detection plane.

During volumetric acquisition, successive projected slices are stacked sequentially. Because these slices are recorded in the fixed detection coordinate system, the inherent obliqueness of the illumination geometry is not preserved in the raw stack. Instead, the dataset represents a sheared or scaled version of the true sample volume.

To reconstruct the correct three-dimensional geometry, an affine transformation is applied to the acquired dataset. This transformation consists of a combination of scaling and shearing operations derived from the known illumination angle. The affine matrix compensates for (i) the projection-induced scaling and (ii) the geometric tilt of the illumination plane, thereby restoring the correct spatial orientation and proportions of the reconstructed volume.

Let the true axial extent of the sample be d , defined along the optical axis of the detection objective. In the present system, a single oblique plane is imaged by scanning the oblique light sheet through the sample using an electrically tunable lens, whereas volumetric acquisition is achieved by translating the sample relative to the light sheet using either galvo-based or mechanical scanning. The sampling interval is determined by the scan step size Δy , and the total scanned extent is given by the number of scan steps N multiplied by Δy .

Because the illumination plane is tilted by an angle α , each recorded frame represents the projection of an oblique sample section onto the fixed camera coordinate system. An axial sample extent d therefore maps to a length $d/\cos(\alpha)$ along the oblique image plane. As the sample is scanned through the light sheet along the y -direction, successive oblique sections are laterally displaced in the recorded image stack, producing a shear distortion. An affine transformation is therefore applied to reconstruct the correct spatial geometry of the volume. A schematic representation of the deskewing process is shown in fig. 28.

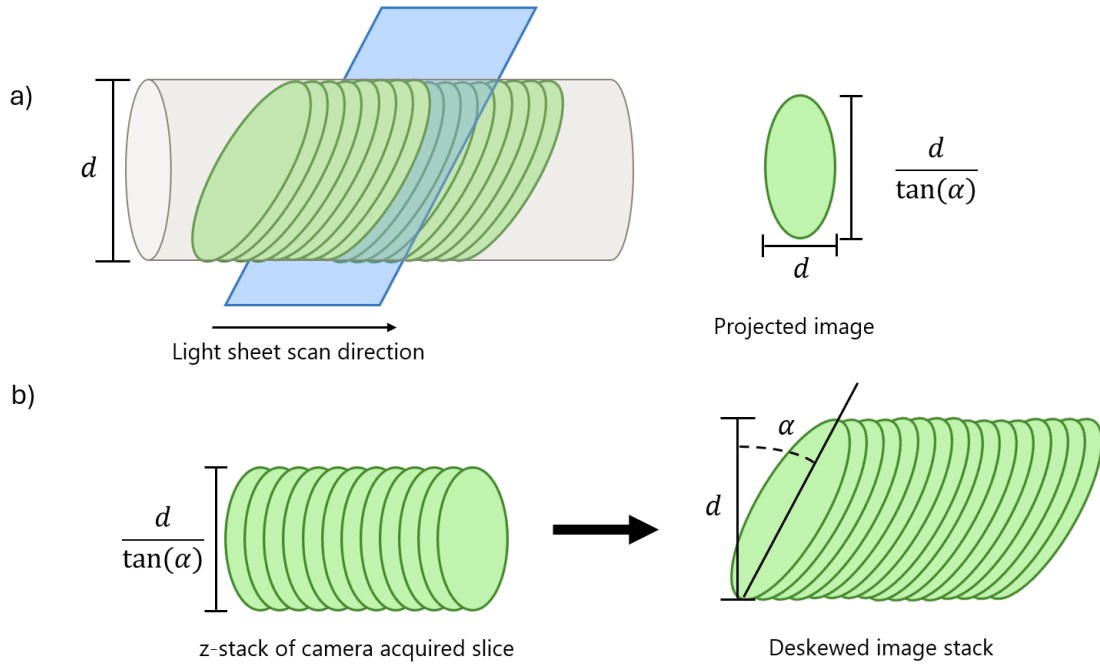


Figure 28. Schematic representation of deskewing performed by affine transform in image stack. a) Imaging geometry and projected image. b) Acquired stack and final deskewed stack. Modified from [46].

Geometrically, a sample height d along the axial direction is projected as a lateral extent $d \tan \alpha$ in the camera y -direction. Let L_x represent the extent along the unchanged in-plane direction and let the total scan range be $L_y = N \Delta y$. The true physical volume is given by $L_x d N \Delta y$. However, the apparent volume in the raw camera stack is given by $L_x (d \tan \alpha) (N \Delta y)$.

If the acquired frames are stacked directly without affine correction, the reconstructed volume becomes geometrically distorted because the oblique acquisition geometry is not preserved. Instead of rotating the entire volume after acquisition, the dataset can be corrected more efficiently by applying a shear

transformation. To recover the true sample volume, the dataset is mapped by an affine transformation onto a larger bounding stack of volume $L_x(N\Delta y + d\tan\alpha)d$, yielding the corrected sample volume $L_x d N\Delta y$. The affine matrix T is,

$$T = \begin{bmatrix} 0 & 1 & 0 \\ \tan(\alpha) & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix} \quad (19)$$

3.8 Speed characterisation

Volumetric imaging speed is fundamentally determined by two factors:

- i. The number of lateral scan steps required to cover the desired length of sample
- ii. The frame acquisition rate

For a fixed camera frame rate, the volumetric imaging rate is determined by the number of image frames required to reconstruct the desired three-dimensional volume. In the present oblique imaging geometry, each frame corresponds to an oblique section of the specimen. The physical field of view of this section depends on the objective magnification. A low-magnification objective captures a larger oblique field of view, allowing a larger volume to be reconstructed with fewer frames. Conversely, a high-magnification objective captures a smaller oblique field of view, requiring more frames to reconstruct the same volume. Thus, low-magnification imaging provides higher volumetric throughput and larger spatial coverage, whereas high-magnification imaging provides improved spatial resolution at the expense of imaging volume and volume rate.

In addition, high-NA objectives require smaller lateral scan steps to adequately sample the higher spatial frequencies resolved by the system and to ensure sufficient overlap between adjacent scanned regions. Consequently, high-NA, high-magnification imaging typically requires a larger number of scan positions to cover the same sample extent, further reducing the achievable volumetric imaging speed compared with lower-magnification, lower-NA imaging.

Consequently, when using the 60x, 1.1 NA objective, the volumetric imaging rate (volumes per second) is reduced compared to the 20x, 0.5 NA objective for the same sample extent. Although the frame rate of the camera may remain unchanged, the total number of frames required to construct a volume increases due to reduced FoV and finer lateral sampling.

In contrast, the 20x objective enables faster volumetric coverage of large sample regions. The larger FoV reduces the number of lateral position required, and the lower NA allows for relatively larger lateral step sizes, decreasing the number of frames per volume. This makes the lower-magnification configuration better suited for rapid mesoscopic imaging where spatial coverage is prioritized over maximum resolution.

3.9 FoV characterization at different magnifications

To evaluate the flexibility of the present system, imaging was performed using two different objective lenses: a 20x, 0.5 NA objective and a 60x, 1.1 NA water-immersion objective. These objectives represent two distinct imaging regimes: mesoscopic-scale imaging with extended FoV and high-resolution subcellular imaging, respectively.

The lateral FoV scales inversely with magnification for a fixed camera sensor size [10]. Consequently, the 20x objective provides a substantially larger FoV compared to the 60x objective. This larger FoV enables imaging of extended biological structures within a single frame, which is advantageous for mesoscopic applications and large tissue samples.

In contrast, the 60x, 1.1 NA objective provides significantly higher lateral and axial resolution due to its larger numerical aperture. However, this increase in magnification reduces the accessible FoV and increases the spatial sampling density required to satisfy Nyquist criteria. As a result, more pixels are allocated per unit area of the sample, and a smaller physical region is imaged per frame.

The choice between the 20x and 60x objectives therefore represents a trade-off between spatial resolution and FoV. The high-NA objective enables superior resolution and improved signal collection efficiency, but at the expense of reduced

FoV. Conversely, the lower-magnification objective enables large-area imaging with moderate resolution.

Importantly, the present system architecture supports both regimes without requiring changes to the remote focusing mechanism, demonstrating its adaptability across different imaging scales [15], [18].

3.10 Resolution estimation

After realisation of the microscope resolution characterization of the system was performed using sub-diffraction fluorescent microbeads imaged with the 60x, 1.1 NA water-immersion objective. The point spread function (PSF) was estimated by extracting the intensity distribution of individual beads and fitting a Gaussian profile to determine the full width at half maximum (FWHM) along each spatial direction as shown in fig 29c.

The measured average lateral resolutions were: $0.52 \pm 0.27 \mu\text{m}$ along the x- direction, $0.58 \pm 0.28 \mu\text{m}$ along the y-direction and the axial resolution was measured to be: $2.57 \pm 0.68 \mu\text{m}$. The lateral resolution (fig 29b) values are comparable to those obtained in our earlier system configuration, indicating that the modifications introduced in the upgraded setup do not significantly degrade transverse imaging performance.

However, the axial resolution exhibited noticeable degradation across the field of view (fig. 29a). Specifically, the axial resolution progressively worsened with increasing imaging depth. In addition, the improvement in axial resolution near the optimal focal plane of the high-NA objective, typically observed as a minimum in the resolution-versus-depth curve [29], was not evident in the present measurements.

The absence of the characteristic axial resolution minimum near the nominal focal plane suggests the presence of depth-dependent spherical aberration. We attribute this primarily to aberrations introduced by the additional 4f telescope incorporated to accommodate the galvanometric scanner. While the telescope was designed to preserve pupil conjugation, residual spherical aberration may arise from imperfect alignment or lens design limitations.

This contribution adds to the intrinsic spherical aberration of the high-NA objective, particularly when imaging through refractive index (RI) mismatched media. Although the objective is equipped with a correction collar to compensate for cover glass thickness and RI mismatch, imperfect adjustment or depth-dependent refractive index variation can introduce additional spherical aberration. The collar setting itself may influence the axial PSF shape at different focal positions.

Spherical aberration scales strongly with numerical aperture and refractive index mismatch. While axial resolution varies approximately inversely with NA, spherical aberration increases rapidly with NA (approximately with a fourth-order dependence on pupil radius)[13]. Consequently, axial resolution degradation becomes more pronounced in high-NA systems, whereas lateral resolution remains comparatively less affected.

Across the axial FoV, the lateral resolution remained approximately uniform. Similarly, along the galvanometric scan direction, no significant variation in lateral resolution was observed. This indicates that the scanning mechanism and pupil utilization remain stable across the FoV and that the dominant aberration primarily affects axial confinement rather than transverse resolution.

To avoid artifacts associated with refractive index mismatch at the coverslip interface, the dataset used for resolution analysis was extracted from a depth of 15 μm above the coverslip. The region immediately adjacent to the coverslip exhibited anomalous behaviour, likely due to refractive index mismatch between the agarose embedding medium and the glass coverslip.

Bead based characterization showed that the system operates near the diffraction-limited resolution of the objective, indicating that the microscope preserves its full-aperture imaging capability. Furthermore, the introduction of the galvanometric scanner and dual-colour imaging did not cause any significant degradation in resolution. Overall, an OPM system capable of oblique-plane imaging with dynamic remote focusing, full-aperture imaging, and optical sectioning, together with multicolour imaging and galvo-based scanning, was realized.

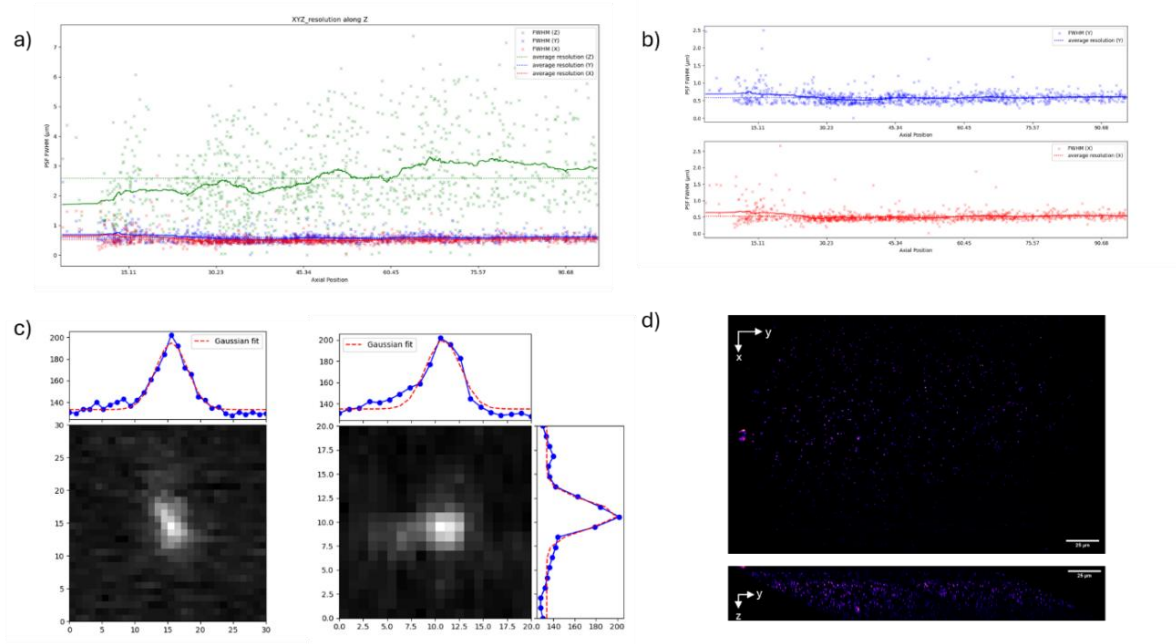


Figure 29. Microbead imaging. (a) Estimation of lateral and axial resolution of microbeads using FWHM of Gaussian fit over intensity distribution. (b) Top panel shows the estimated lateral resolution along y direction (Galvo scan direction) and bottom panel shows the lateral resolution along x direction. (c) Left panel shows the intensity distribution along axial direction and the Gaussian fit over it along z direction, right panel shows the intensity distribution laterally and Gaussian fit both over x and y direction. (d) Top panel shows the maximum intensity projection of beads in x-y plane and bottom panel shows the maximum intensity projection of bead in y-z plane.

This chapter presented the design and implementation of a full-aperture oblique plane microscope developed using commercially available optical components. The system combines dynamic remote focusing, synchronized rolling shutter detection, galvanometric scanning, and multicolour imaging capability within a single-objective architecture. By eliminating the tilted detection geometry employed in conventional OPM systems, the microscope preserves the full numerical aperture of the primary objective while maintaining the optical sectioning advantages of light-sheet microscopy.

The use of an electrically tunable lens for dynamic remote focusing enabled simultaneous scanning and de-scanning of the oblique illumination plane, allowing tilted-plane imaging without mechanical rotation of the detection path. Synchronization of the scanned light sheet with the rolling shutter of the sCMOS camera provided confocal-like rejection of out-of-focus fluorescence, improving image contrast and optical sectioning. Furthermore, the absence of a tilted tertiary

objective simplifies alignment and provides greater flexibility in objective selection compared to conventional OPM architectures.

The following chapter presents the experimental validation of the developed system using different biological samples.

4. Applications in biological imaging

4.1 Introduction

This chapter presents imaging experiments conducted to evaluate the performance of the microscope under practical biological imaging conditions. A key feature that distinguishes our microscope from conventional OPM systems is that it does not rely on a mechanically tilted detection geometry. This provides greater flexibility when switching between objective lenses of different magnifications, without significant loss of imaging performance or the need for optical realignment. In addition, the system retains full-aperture capability. This can provide an important practical advantage for biologists, as it enables imaging of the same sample at different magnifications and fields of view. The experiments were therefore designed to assess whether image quality and optical sectioning could be maintained across different objective configurations while preserving applicability to real biological specimens.

To evaluate this capability, imaging experiments were performed using an Olympus LUMFL 60x/1.10 NA objective and an Olympus UMPLFN 20x/0.50 NA objective. In addition to bead-based testing, imaging was carried out in biological samples to verify that performance was not compromised under realistic specimen conditions. The main specimens imaged were a commercially available 16 μm thick cryostat section of mouse kidney with immunofluorescence staining (FluoCells Prepared Slide #3, Invitrogen, United States), zebrafish embryos at 3–5 day post fertilization (dpf), prepared *Drosophila* brain slides, organoids, mouse embryos, and bovine oocytes mounted on a conventional flat microscope slide. Zebrafish embryos from the transgenic lines Tg(kdrl:GFP); Tg(gata1:dsRed) were imaged. In this notation, Tg denotes a transgenic line, kdrl (kinase insert domain receptor like) drives expression in vascular endothelial cells, GFP denotes green fluorescent protein, gata1 (GATA-binding factor 1) is associated with blood cells, and dsRed denotes red fluorescent

protein. The developmental age of the embryos is expressed in days post-fertilisation (dpf).

Imaging was performed at two stages in the development of the microscope. In the initial system, imaging was single-colour and scanning was performed using a translational stage. In the upgraded system, dual-colour capability was introduced, and scanning was performed using a combined galvo and translational-stage approach. Consequently, imaging with the initial system configuration was limited to the kidney sample and zebrafish embryos, whereas the upgraded system was used to image all the specimens listed above. The initial system further lacked multicolour imaging capability and galvo-based scanning and was therefore restricted to samples labelled with a single fluorophore. Although live zebrafish embryos were imaged, real-time dynamics could not be recorded. By contrast, the upgraded system enabled imaging of a wider range of specimens and provided greater experimental flexibility. The remainder of this chapter presents the imaging results obtained with both system configurations and assesses their performance across different sample types, with the discussion divided into low-magnification and high-magnification imaging sections.

4.1 Low-Magnification Imaging

4.1.1 Prepared-Slide Imaging

One of the features that makes OPM particularly attractive, compared with many conventional light-sheet microscope implementations, is its compatibility with standard sample-mounting formats such as optical slides and petri dishes. This avoids the need for specialised mounting geometries that are often required in orthogonal light-sheet arrangements. For this reason, the first imaging experiments were performed on a mouse kidney sample (fig. 30). This is a widely used specimen in fluorescence microscopy and therefore provides a useful basis for comparison with confocal imaging. The mouse kidney specimen consists of glomeruli and convoluted tubules. In FluoCells Prepared Slide #3, elements of the glomeruli and convoluted tubules are labelled with Alexa Fluor 488 wheat germ agglutinin (W-

11261), while filamentous actin, particularly in the glomeruli and brush border, is labelled with Alexa Fluor 568 phalloidin (A-12380). The slide also contains DAPI as a nuclear stain. However, the present microscope was configured for two-colour imaging with 488 nm and 568 nm channels, and therefore only the Alexa Fluor 488 and Alexa Fluor 568 signals were used in these experiments [53].

The kidney specimen is prepared as a 16 μm cryostat section. For the 20x/0.50 NA configuration, the theoretical detection axial resolution for the fluorophores used in this sample is approximately 4–5 μm . The section thickness is therefore several times larger than the axial extent of a single optical section, making it a useful specimen for qualitatively assessing optical sectioning performance and out-of-focus background rejection.

Volumetric imaging of the mouse kidney sample was carried out over a scan length of 250 μm , with a plane spacing of 0.4 μm , using a 2048 x 256 pixel sub-region of the detector. This configuration provided a larger field of view than the 60x objective, but at the expense of spatial resolution and optical sectioning performance. Nevertheless, the resulting dataset still provided a clear three-dimensional representation of the specimen.

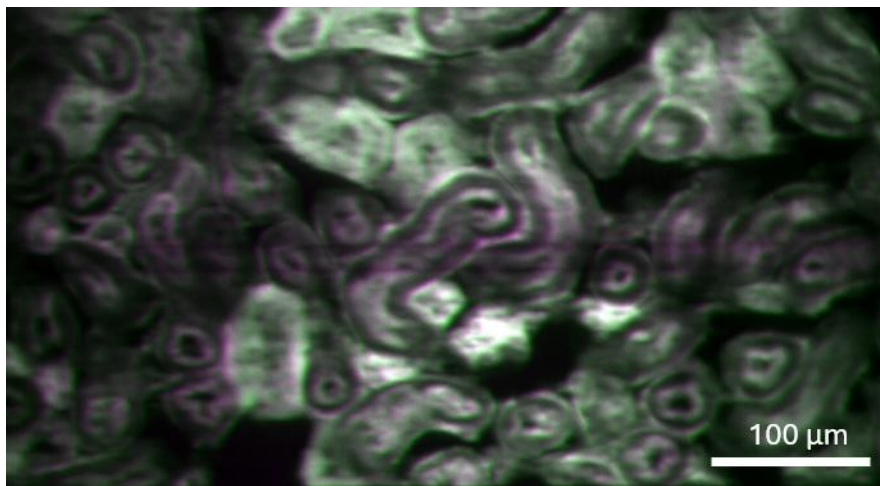


Figure 30. Large-field-of-view volumetric image of the mouse kidney specimen acquired with the 20x/0.50 NA objective.

4.1.2 Full organism imaging

Zebrafish are one of the most widely used vertebrate model organisms in biological and biomedical research. Their rapid external development, early-stage optical transparency, and genetic tractability make them particularly well suited for *in vivo* imaging. In addition, they are easy to maintain in the laboratory, produce large numbers of embryos, and have a short developmental cycle. Zebrafish are therefore widely used in studies of vascular development, blood flow, neural activity, disease models, and drug screening, making them a valuable biological specimen for evaluating the imaging performance of the present microscope.

Zebrafish embryos aged 3–5 dpf, Tg(*kdr*:GFP); Tg(*gata1*:dsRed), with fluorescent labelling of the vasculature and blood cells, respectively, were used for imaging. The embryos were anaesthetised with tricaine solution and mounted in agarose on a coverslip attached to a custom 3D-printed holder. Small channels were formed in the agarose to hold the embryos in water during imaging, thereby maintaining their position during acquisition and aligning them with the galvo scanning direction. Embryonic pigmentation was suppressed by treatment with 1-phenyl-2-thiourea (PTU).

As the zebrafish embryo is a mesoscopic specimen with anatomical features distributed over a relatively large spatial extent, a large field of view is advantageous for observing different regions of the sample simultaneously. For this reason, low-magnification imaging was performed using the 20x/0.50 NA water-dipping objective. This configuration provided a sufficiently large field of view for imaging extended regions of the embryo, while maintaining optical sectioning and axial resolution suitable for resolving cellular-scale structures.

Using this configuration, images of both the head and tail regions were acquired. In the large-field images, motion artefacts were visible in the region of the heart, as the data were acquired at 20 fps with a scan step size of 0.5 μm . To capture the full zebrafish embryo, a combined galvo and translational-stage scanning approach was employed. Adjacent image sections were acquired with the 20x objective and subsequently stitched using custom software to reconstruct the full-embryo image

(fig. 31). A slight truncation at the lower part of the head was observed in the reconstructed image, which was attributed to a small misalignment between the embryo orientation and the galvo scan direction.

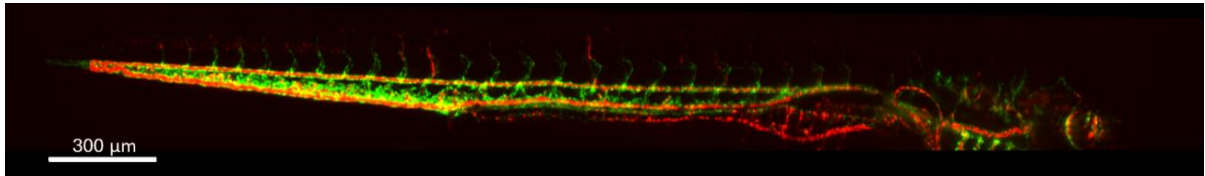


Figure 31. Full-organism image of the zebrafish embryo reconstructed by stitching adjacent 20x image tiles acquired using combined galvo and translational-stage scanning.

4.1.3 High speed dynamic imaging

In addition to whole-embryo imaging, the cardiac region was specifically imaged to evaluate the dynamic performance of the microscope. This enabled us to assess the performance of galvo-based volumetric imaging in a dynamic biological specimen, mainly blood flow driven by the beating zebrafish heart was imaged. Since the zebrafish heart beats at approximately 3 beats per second, a volumetric imaging rate of at least 6 volumes per second was taken as a practical minimum requirement for resolving heartbeat dynamics. This therefore provided a useful target for evaluating the temporal performance of the system.

For volumetric imaging, the ETL scanned the oblique FoV synchronously with the camera acquisition, while the galvo stepped laterally after completion of each ETL scan. In this way, the ETL defined the scan along the oblique direction, and the galvo provided sequential lateral stepping to build the full volume. All these events were synchronously controlled using a National Instruments board. Because the ETL modulation rate was matched to the camera frame rate, increasing the frame rate directly increased the achievable volume rate. To enable higher frame rates, the camera sensor height was reduced from 1024 pixels to a 128-pixel sub-region, allowing acquisition at 150 fps.

A field of view of 200 μm was imaged with a step size of 6.67 μm , yielding a volumetric imaging rate of 5 volumes per second. This represented the maximum volumetric imaging rate that could be achieved while still preserving usable image

quality over the full heart. Although higher volume rates could in principle be obtained by increasing the lateral step size, this led to under sampling of the volume and loss of image detail due to missed sampling points within the specimen. In addition, increasing the ETL scan frequency beyond 150 Hz resulted in imperfect synchronisation due to ETL response to a sawtooth input, which also introduced ETL-related aberrations and further degraded image quality. The 200 μm field of view therefore enabled full-heart imaging, but at the cost of a volume rate slightly below that required to fully resolve the heartbeat dynamics. A maximum-intensity projection along the z-axis was generated for each time point, as shown in the fig. 32.

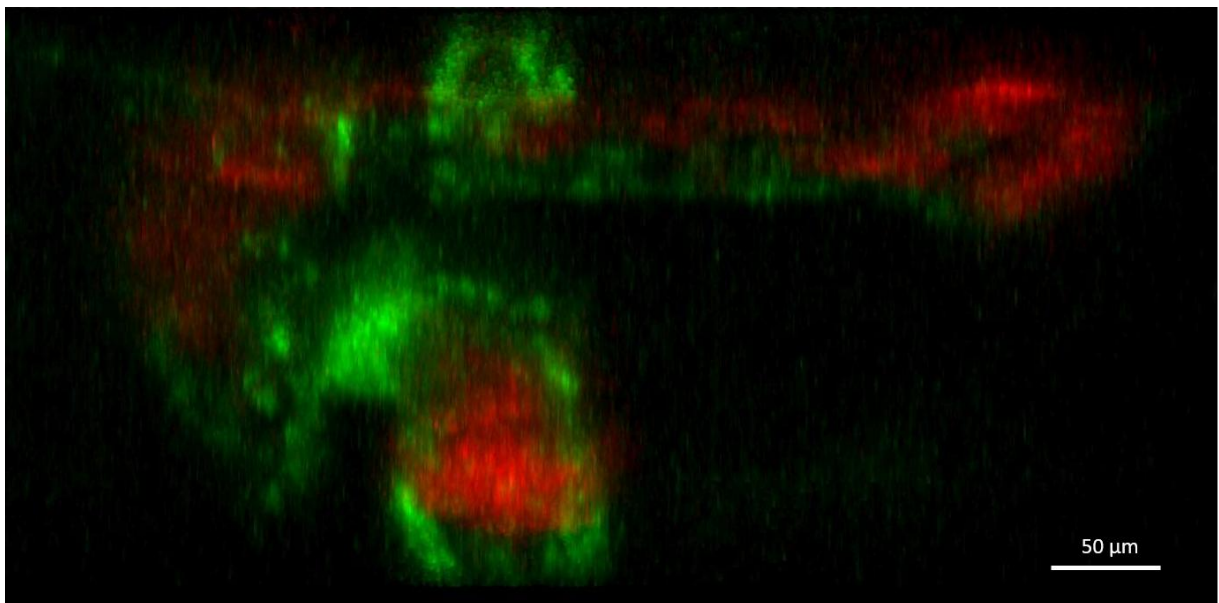


Figure 32. Low-magnification large-field-of-view image of zebrafish heart acquired with the 20x/0.50 NA objective.

4.1.4 Imaging of additional biological samples

The versatility of the low-magnification configuration was further demonstrated using additional specimens prepared in standard mounting formats. Prepared *Drosophila* brain slides (fig. 33a) with dual fluorescent labelling were imaged over a scan range of 640 μm with a step size of 0.25 μm . Prepared bovine oocyte slides with dual fluorescent labelling (fig. 33b-d) were also imaged over a scan range of 350 μm with a step size of 0.5 μm , allowing cells at different developmental stages to be observed.

To evaluate the effect of ETL scan frequency on image quality, imaging was performed over a range of ETL modulation frequencies from 10 Hz to 50 Hz. Across this range, no significant difference in image quality was observed, and the ETL maintained good synchronisation over the full field of view. Although the exposure time was adjusted slightly from the nominal synchronised value to improve image brightness, no noticeable degradation in signal-to-noise ratio or structural detail was observed. These results indicate that the system can maintain stable volumetric imaging performance at ETL modulation frequencies up to 50 Hz.

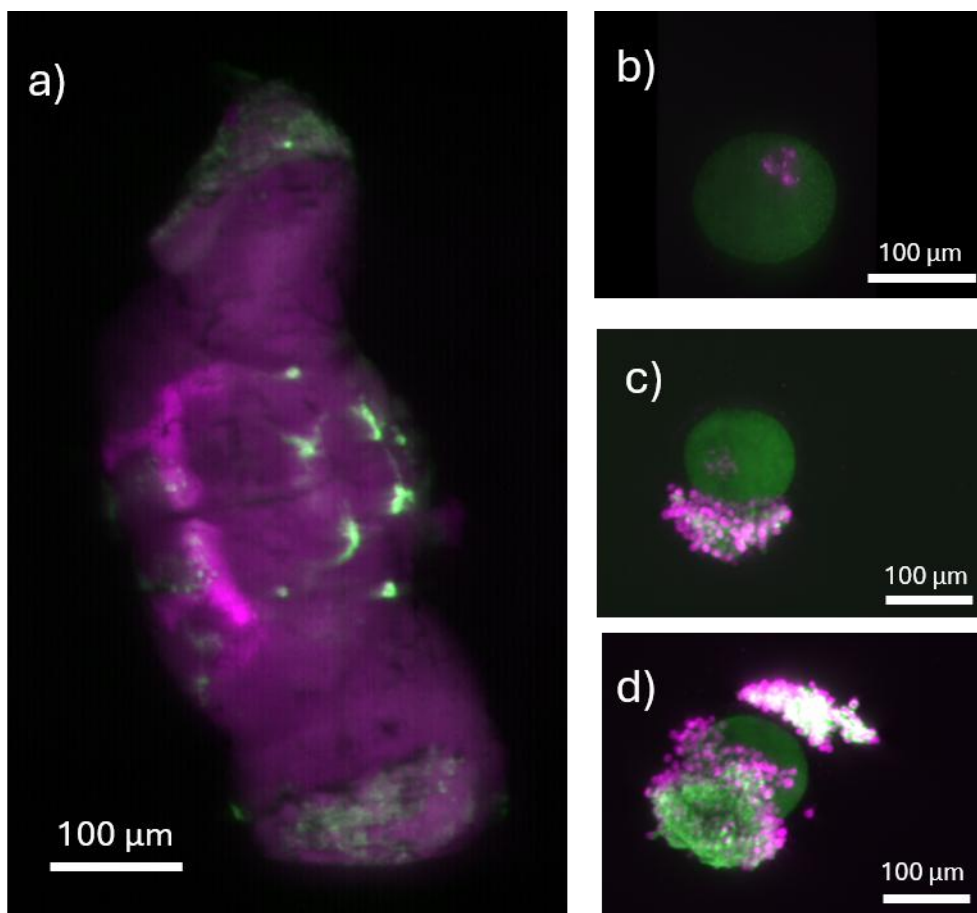


Figure 33. Images of additional biological specimens acquired with the low-magnification configuration a) *Drosophila* brain, b-d) bovine oocyte.

4.2 High-Magnification Imaging

To complement the low-magnification imaging results presented above, the same specimens were also imaged using the Olympus LUMFL 60x/1.10 NA water-dipping objective. Whereas the 20x/0.50 NA configuration provided a larger field of view for imaging extended regions of the sample, the 60x/1.10 NA configuration was used to assess the high-resolution imaging capability of the microscope. This higher-magnification configuration enabled improved visualisation of fine structural features. Imaging the same biological specimens at both magnifications also allowed a direct comparison between large-field imaging and high-resolution imaging without requiring realignment of the optical system.

4.2.1 Prepared-Slide Imaging

In addition to the low-magnification imaging described above, the mouse kidney specimen was also imaged using the 60x/1.10 NA objective (fig. 34). At this higher magnification, finer structural details within the glomeruli and convoluted tubules could be resolved more clearly. The higher numerical aperture of the 60x objective also provided stronger optical sectioning, resulting in improved contrast and clearer visualisation of features at different depths within the specimen. As expected, this improvement in resolution was accompanied by a reduction in field of view relative to the 20x configuration. These measurements therefore served as a useful comparison with the 20x data, demonstrating that the microscope could be operated at higher resolution while maintaining good image quality in a standard slide-mounted specimen.

Volumetric imaging of the kidney sample was performed over a scan range of 200 μm , with a plane spacing of 0.2 μm , using the 60x/1.10 NA objective. The resulting data showed improved structural detail compared with the low-magnification configuration, particularly in regions containing fine actin-labelled features. These results demonstrate that the system is capable of high-resolution volumetric imaging in biologically relevant samples while preserving good optical sectioning performance.

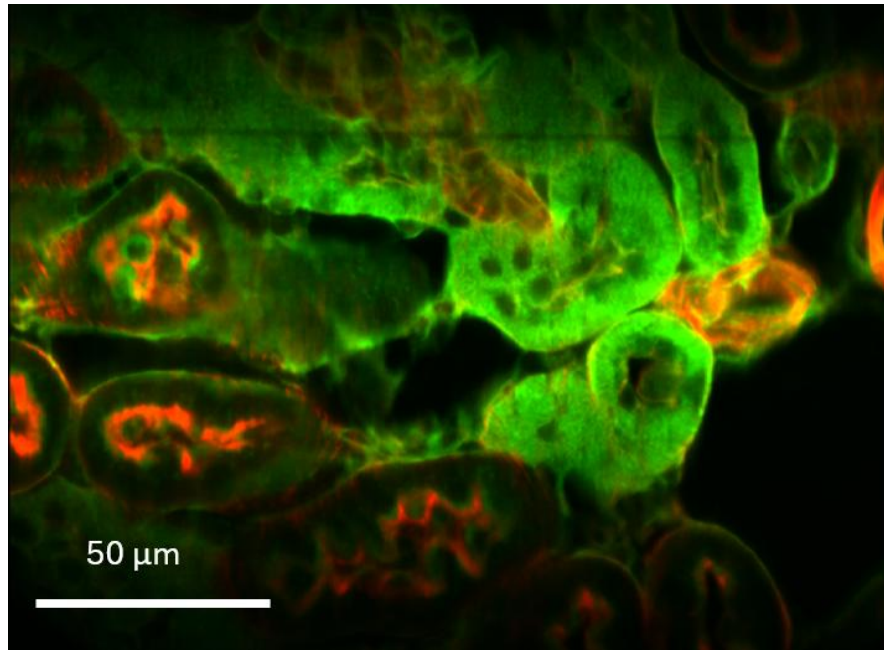


Figure 34. High-resolution image of the mouse kidney slide acquired with the 60x/1.10 NA objective, showing fine structural details.

4.2.2 High-Resolution Imaging of Selected Regions

In the high-magnification configuration, zebrafish embryos were imaged using the 60x/1.10 NA objective in order to resolve finer vascular and cellular detail (fig. 35). While the smaller field of view prevented imaging of the full embryo in a single acquisition, the 60x configuration enabled more detailed examination of selected regions of interest, including different sections of the head, tail, and cardiac region. This provided a direct comparison with the low-magnification data, in which larger anatomical regions could be visualised, but with lower spatial resolution.

The higher numerical aperture of the 60x objective allowed improved visualisation of the vasculature and blood-labelled structures in the *Tg(kdrl:GFP); Tg(gata1:dsRed)* embryos. In particular, smaller vessels and finer morphological features could be distinguished more clearly. These results demonstrate that the microscope can provide high-resolution imaging of live zebrafish embryos while retaining the same practical sample preparation used for low-magnification imaging.

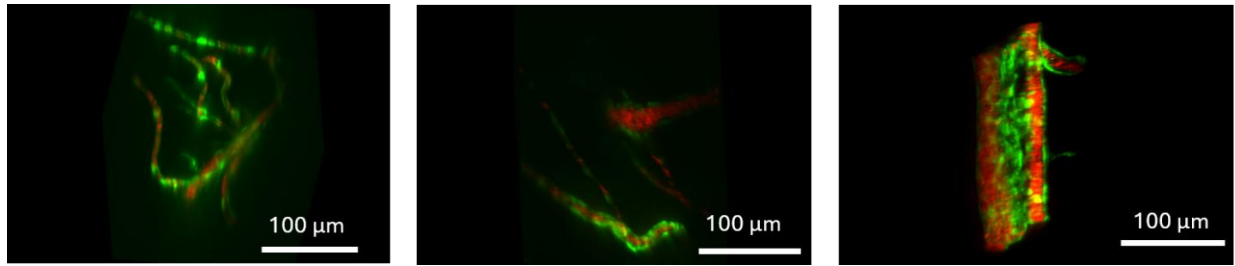


Figure 35. High-resolution image of vasculature and blood flow in the head and tail regions of the zebrafish embryo, acquired with the 60x/1.10 NA objective.

4.2.3 High-Speed Volumetric Imaging

A more detailed evaluation of the high-magnification performance at high speed was carried out by imaging the zebrafish cardiac region using the 60x/1.10 NA objective. In contrast to the 20x configuration, which provided a larger field of view over the full heart and surrounding blood vessels, the 60x configuration enabled higher-resolution imaging of specific regions of the beating heart. This made it possible to examine finer structural detail, although over a reduced spatial extent.

Volumetric imaging of the cardiac region was performed over a scan range of 100 μm with a scan step size of 5 μm which enabled imaging at a rate of 7.5 volume per second. Similar to 20x configuration images were acquired at 150 fps with a reduced camera sensor height at 128 pixels. The higher-resolution data provided clearer visualisation of cardiac morphology mainly the cardiac wall than the low-magnification configuration as shown in fig. 36. However, the blood-flow signal was strongly blurred owing to the rapid motion of the blood cells during acquisition, which introduced substantial motion blur. As expected, this increase in spatial resolution was accompanied by a reduced field of view and a more restricted imaging volume. Together, the 20x and 60x measurements illustrate the flexibility of the system in balancing field of view, spatial resolution, and temporal performance, making it suitable for a range of biological imaging applications.

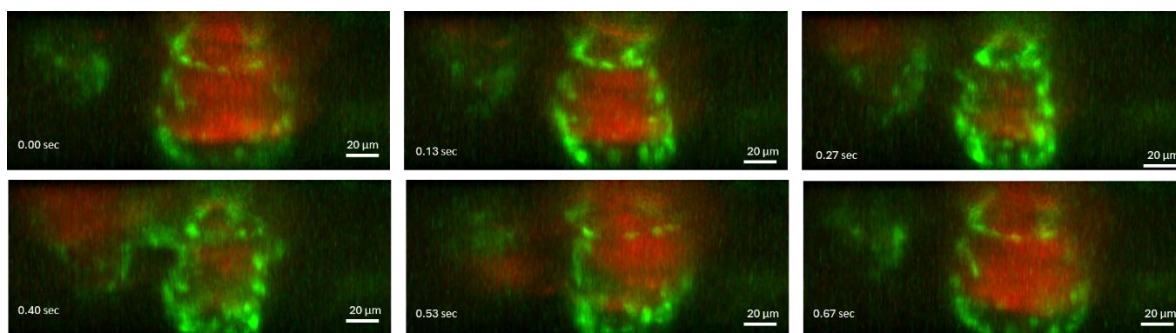


Figure 36. High-speed imaging of the 3 dpf zebrafish embryo beating heart over one full cardiac cycle.

4.2.4 High-Magnification Imaging of Additional Biological Samples

The versatility of the high-magnification configuration was further demonstrated using additional biological specimens. Prepared *Drosophila* brain slides were imaged using the 60x/1.10 NA objective, allowing more detailed visualisation of labelled structures than in the corresponding low-magnification data. This showed that the microscope could maintain high-resolution optical sectioning performance in a conventional 2D prepared slide while using dual-colour imaging.

Prepared bovine oocyte slides (fig. 37a,b) were also imaged using the 60x/1.10 NA objective in order to resolve finer subcellular and morphological detail than was accessible at 20x magnification. The higher-resolution images enabled clearer observation of structural differences between oocytes at different developmental stages. Imaging was performed over a scan range of 100 μm with a step size of 0.25 μm , demonstrating that the system could maintain high image quality in slide-mounted biological specimens at high magnification.

In addition, 2D organoid slides were imaged using the 60x/1.10 NA objective. Relative to the 20x configuration, the high-magnification data revealed finer internal structural detail and provided improved visualization of local morphology. The organoid samples (fig. 37c) were imaged over a scan range of 100 μm with a step size of 0.25 μm , further demonstrating the versatility of the microscope across different sample types and imaging scales.

Mouse embryo samples (fig. 37d) were also imaged at high magnification using the 60x/1.10 NA objective. These specimens were mounted in a well-like structure containing aqueous medium, providing a useful demonstration of the compatibility of

the system with non-slide-mounted biological samples. Imaging was carried out over a scan range of 100 μm with a step size of 0.25 μm . The resulting data showed that the microscope could achieve high-resolution imaging in more complex sample environments while retaining the same general imaging architecture.

For all of these acquisitions, the camera sensor area was 1024 x 2048 pixels for each colour channel. All image volumes presented in this chapter were reconstructed using an affine transformation. The transformation parameters were selected according to the magnification and numerical aperture of the objective used for acquisition, as these influence the obliqueness of the image plane.

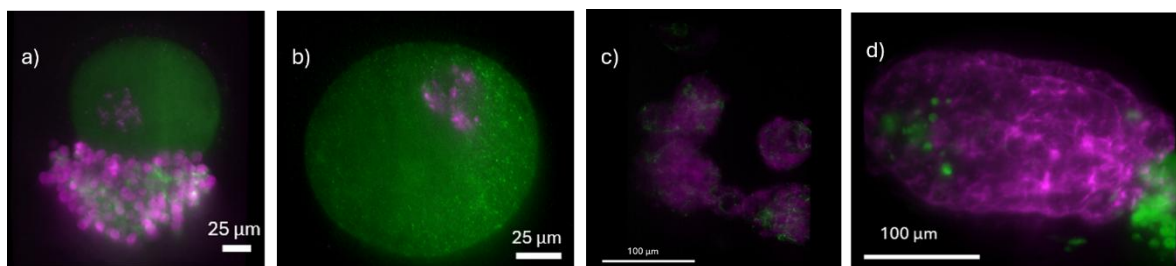


Figure 37. Representative high-resolution images of additional biological samples like a,b) bovine oocytes, c) organoids and d) mouse embryo acquired with the 60x/1.10 NA objective.

This chapter has demonstrated the performance of the microscope in practical biological imaging through experiments on a range of representative specimens. Imaging was performed on mouse kidney sections, zebrafish embryos, *Drosophila* brain slides, bovine oocytes, organoids, and mouse embryo samples, thereby showing that the system is applicable to both prepared specimens and live biological samples. The results confirm that the architecture remains compatible with conventional sample mounting formats, which is a significant practical advantage over many conventional light-sheet implementations.

A central aim of this chapter was to evaluate whether imaging performance could be maintained while switching between objective lenses of different magnification. The results show that the 20x configuration provides a larger field of view and is well suited for imaging extended specimens and dynamic volumetric processes, whereas the 60x configuration enables improved spatial resolution and stronger optical sectioning for more detailed structural imaging. Although this introduces the expected trade-off between field of view, temporal performance, and spatial

resolution, the ability to access both regimes within the same microscope, without requiring realignment, represents a key strength of the system. Furthermore, the upgraded implementation, incorporating galvo-based scanning and dual-colour imaging, extended the versatility of the platform and enabled imaging of a wider range of biological specimens. Taken together, these results validate the microscope as a flexible and practically useful platform for multi-scale biological imaging.

5. Conclusion

This thesis presented the development, analysis, and experimental validation of a novel oblique plane microscope based on dynamic remote focusing as a flexible alternative to conventional OPM architectures. In contrast to traditional implementations that rely on tilted detection geometries and relay-based remote refocusing, the proposed system enables a simpler, more cost-effective implementation using off-the-shelf components, while retaining full-aperture capability and delivering practical imaging performance across a range of biological imaging conditions. The work presented in this thesis demonstrates that the microscope is compatible with standard sample-mounting formats and can image a variety of biologically relevant specimens, including tissue sections, live zebrafish embryos, prepared slides, organoids, and embryo samples, thereby confirming its versatility in realistic experimental settings. In this work, it was demonstrated that imaging can be performed at both low and high magnification without the need for optical realignment, allowing the same system to support both large-field imaging of extended specimens and high-resolution imaging of selected regions of interest without any significant degradation of performance. The introduction of dual-colour imaging and galvo-based scanning in the upgraded implementation further extended the experimental capability of the system, enabling a broader range of specimens with dual fluorescent labelling and dynamic biological processes to be investigated. At the same time, the results have shown that the present system is primarily limited by the performance of the electrically tunable lens, particularly with respect to synchronization, aberrations, and achievable volumetric imaging speed. Future variants of this microscope will focus on improving the remote-focusing element and maintaining overall system synchronization during high-speed imaging; this can be achieved by incorporating better adaptive optical components, such as deformable mirrors, which are faster and offer more stable wavefront control. Such

developments could further enhance image quality, reduce aberrations, and improve the system's temporal and spatial performance. Overall, this thesis establishes dynamic remote focusing as a promising direction for developing more accessible, flexible, and practically useful oblique-plane microscopy systems for biological imaging.

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