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Design and Development of a Reflectance Confocal Microscope for Non-Invasive Imaging of Skin Lesions

TESI DI LAUREA MAGISTRALE IN
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Abstract in English

To assess the nature of a suspicious skin lesion, in the vast majority of cases the clinical pathway after dermoscopic examination leads to a biopsy, an invasive surgical procedure that leaves a scar, causes patient discomfort, involves significant healthcare costs, and in many cases turns out to be unnecessary, as it frequently reveals that the lesion is benign. This thesis presents the design and realization of a reflectance confocal microscope, an instrument capable of non-invasive cellular-resolution imaging beneath the skin surface for dermatological imaging and diagnosis. In this work, the full optical and electronic architecture was designed, assembled, and validated in transmission, including scanning synchronization, alignment procedures, and a dedicated reconstruction and visualization pipeline. The reflectance return path is planned as future work to enable confocal detection and complete clinical-grade imaging. The resulting prototype successfully demonstrated stable scanning and image reconstruction on calibration targets and biological specimens in transmission mode, providing a solid basis for completing reflectance-confocal alignment and subsequent *in vivo* validation.

Keywords: Reflectance confocal microscopy, Non-invasive skin imaging, Dermatological diagnostics, Cellular-resolution imaging

Abstract in Italiano

Per valutare la natura di una lesione cutanea sospetta, nella grande maggioranza dei casi il percorso clinico dopo l'analisi dermoscopica porta a una biopsia, una procedura chirurgica invasiva che lascia cicatrici, è causa di disagi per i pazienti, comporta costi sanitari significativi e, in molti casi, risulta non necessaria, poiché spesso rivela che la lesione è benigna. Questa tesi presenta la progettazione e la realizzazione di un microscopio confocale in riflessione, uno strumento in grado di produrre immagini della pelle con risoluzione cellulare e in maniera non invasiva, per applicazioni diagnostiche di dermatologia. In questo lavoro è stata progettata e assemblata l'architettura ottica ed elettronica del sistema e ne sono state validate, in modalità di trasmissione, la sincronizzazione degli scanner, le procedure di allineamento e la pipeline per l'acquisizione dei dati, la ricostruzione e la visualizzazione delle immagini. Il prototipo è stato testato su target di calibrazione e su campioni biologici, mostrando una scansione stabile e ricostruzioni affidabili. Il percorso di ritorno in riflessione è demandato a lavori futuri per abilitare l'acquisizione confocale e completare il sistema per l'imaging *in vivo*.

Parole chiave: Microscopia confocale in riflessione, Imaging cutaneo non invasivo, Diagnostica dermatologica, Imaging a risoluzione cellulare

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1 | Introduction

1.1. Clinical Motivation and Background

Over the past decade, the global incidence of skin cancer has steadily increased, making it one of the most prevalent malignant tumors worldwide. Recent global cancer statistics report more than 1.2 million new cases of non-melanoma skin cancer (NMSC), mainly basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), and around 320,000 cases of melanoma each year [1]. While melanoma represents the most aggressive and life-threatening form, NMSC accounts for a substantial share of the global mortality burden, with more than 60,000 deaths from NMSC and approximately 57,000 deaths from melanoma reported for 2020 [1]. Projections also indicate that melanoma incidence is expected to continue increasing over the coming decades, further strengthening the need for scalable prevention and early-diagnosis strategies [2]. By 2021, the global burden of skin cancer further increased, reaching approximately 2.2 million new cases overall. Among these, melanoma accounted for roughly 300,000 cases, while NMSC accounted for about 1.9 million cases [3]. In 2022, updated global estimates reported a further increase in both incidence and mortality: approximately 331,000 new melanoma cases and 58,000 melanoma-related deaths were registered worldwide, while NMSC accounted for about 1,234,000 new cases and 69,000 deaths [4, 5]. The time evolution of the age-standardized incidence rates for melanoma and NMSCs is shown in Fig. 1.1.1. This rising trend is driven by a combination of environmental, demographic, and clinical factors. Increased ultraviolet (UV) exposure is one of the most significant contributors: solar radiation in the UVA and UVB ranges is a recognized carcinogenic agent for humans [6]. At the biological level, UVA and UVB promote carcinogenesis through a combination of direct DNA damage (particularly UVB-induced photoproducts) and indirect oxidative stress mechanisms, ultimately increasing mutational load in epidermal cells [7]. Epidemiological and mechanistic evidence further supports a strong association between cumulative UV exposure and the incidence of BCC, SCC, and melanoma, especially in populations with high ambient UV indices or behaviors involving intense intermittent sun exposure [8]. Population aging also plays a crucial role in the rising incidence of skin cancer. As people age, the cumulative burden of DNA damage increases, while cellular repair efficiency declines. Older adults therefore account for the majority of new skin cancer diagnoses, reflecting both biological aging and the demographic shift toward older populations worldwide [9, 10]. To quantify trends across populations with different age structures, age-standardized incidence rates (ASIR) and age-standardized mortality rates (ASMR) are commonly used, enabling fair comparisons between countries over time [11]. Global ASIR data indicate a marked increase in skin cancer incidence among older adults from 1990 to 2021: the ASIR for basal cell carcinoma increased by approximately 62.8%, while that for squa-

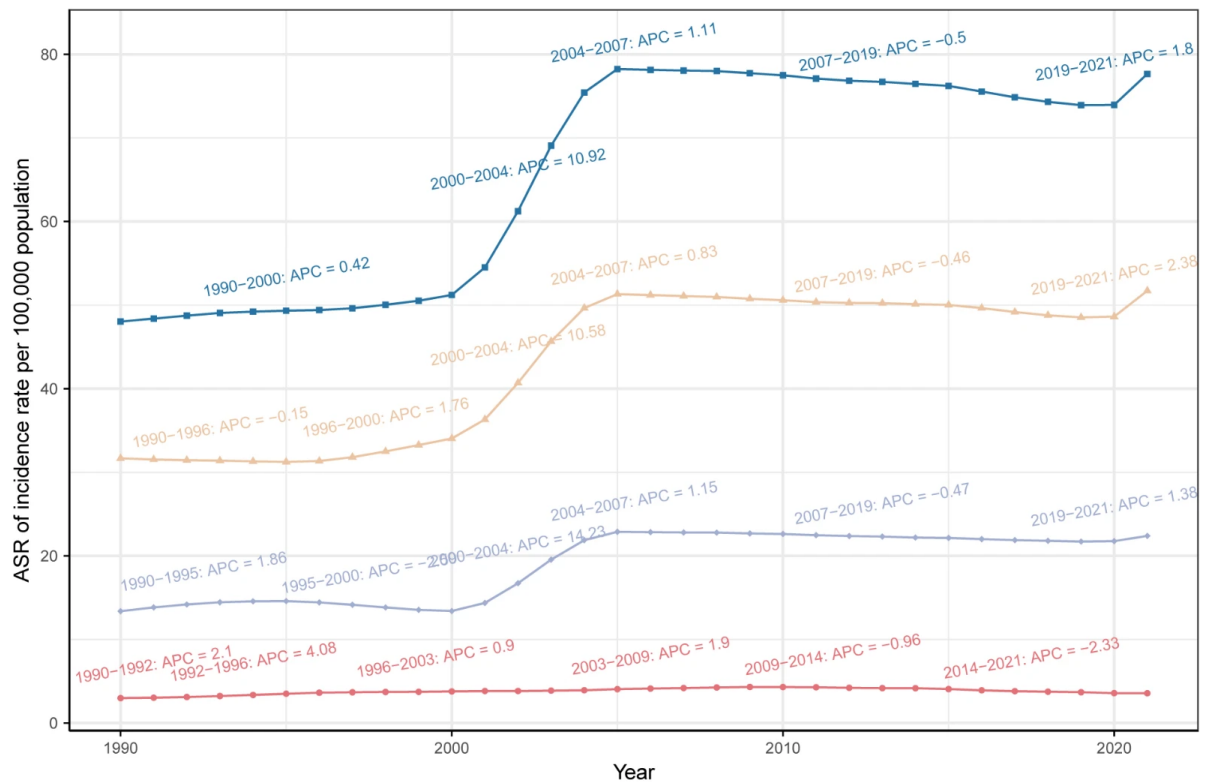
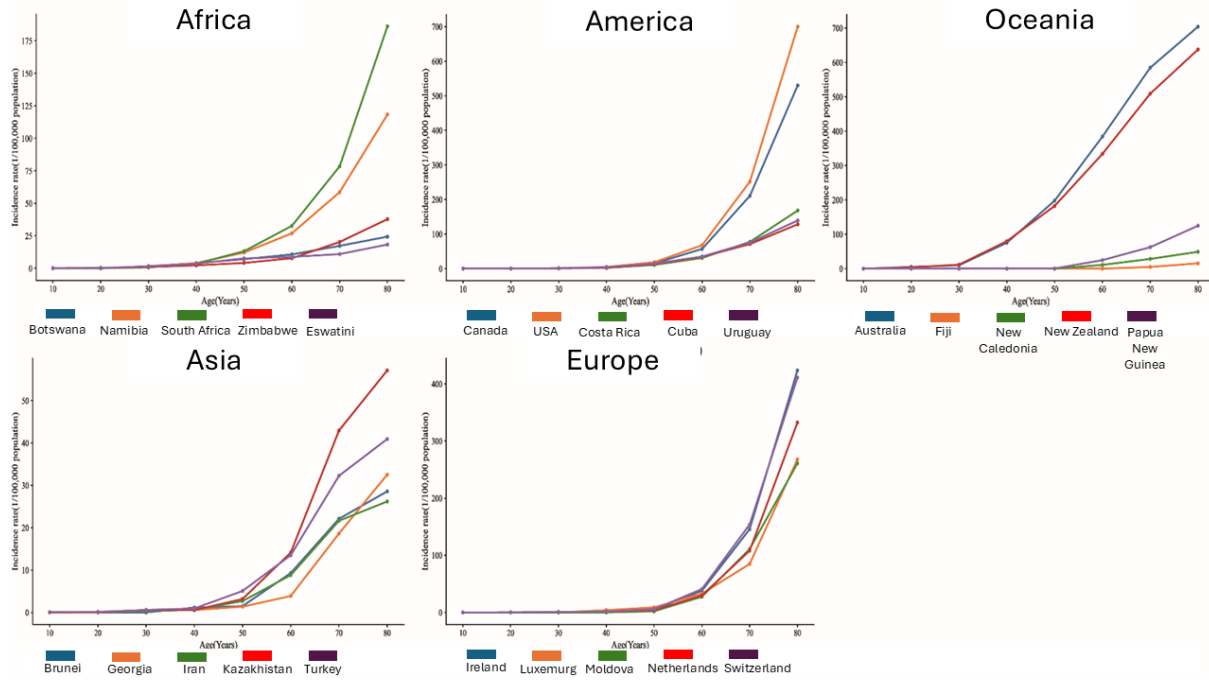
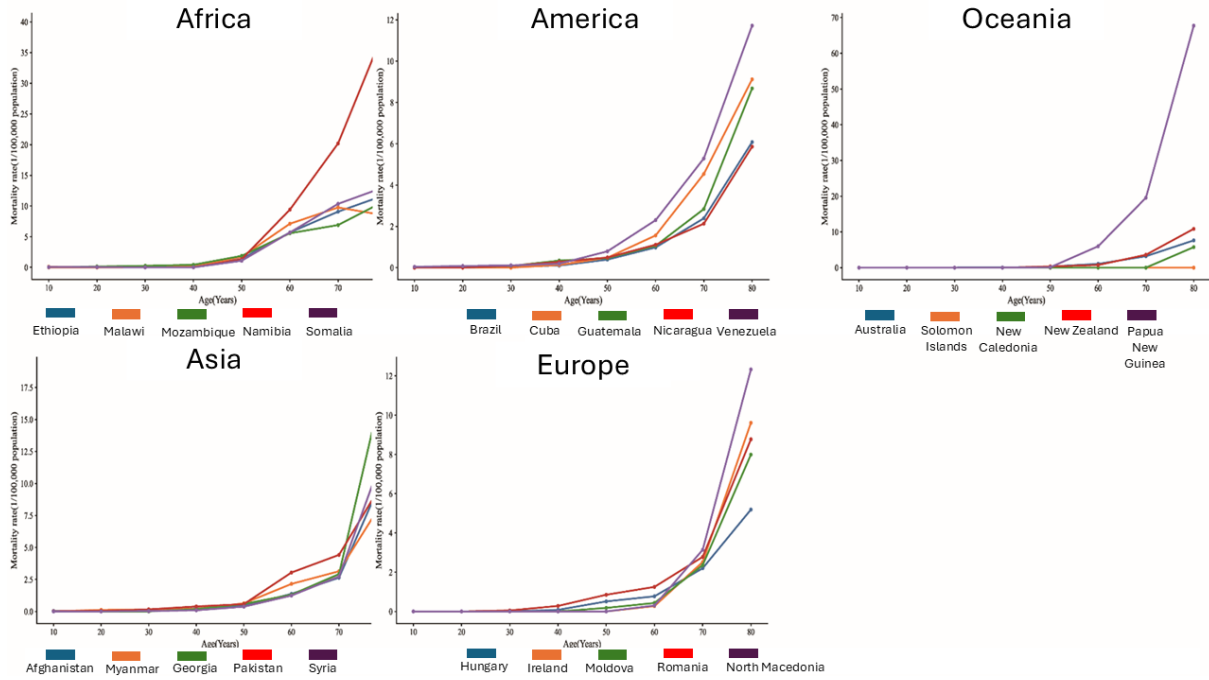


Figure 1.1.1: Trends in age-standardized incidence rate (ASIR) for skin cancers from 1990 to 2021. **Lines:** overall skin cancers (blue), melanoma (orange), basal cell carcinoma (violet), squamous cell carcinoma (red). Adapted from [3].

ous cell carcinoma rose by around 67.4%, highlighting the substantial impact of aging on the global skin cancer burden [12]. Figures 1.1.2 and 1.1.3 show these age-specific ASIR and ASMR patterns for NMSC and melanoma across different countries grouped by continent. Finally, enhanced detection and screening programs have contributed to the observed rise in incidence [14]. Wider clinical adoption of dermoscopy, together with public awareness campaigns and improved access to dermatological care, has led to earlier and more frequent detection of suspicious lesions. In parallel, international efforts to standardize dermoscopic terminology and diagnostic criteria have supported broader and more consistent use in clinical practice [15]. Although part of the observed increase reflects improved surveillance rather than a purely biological rise in disease prevalence, it underscores the growing clinical and societal impact of skin cancer worldwide. Beyond the global trend, substantial regional variations in skin cancer incidence and mortality have been documented, reflecting differences in genetic background, socioeconomic development, and access to healthcare services [13]. Recent analyses, reported in Fig. 1.1.4, indicate that Australia and New Zealand continue to exhibit among the world's highest



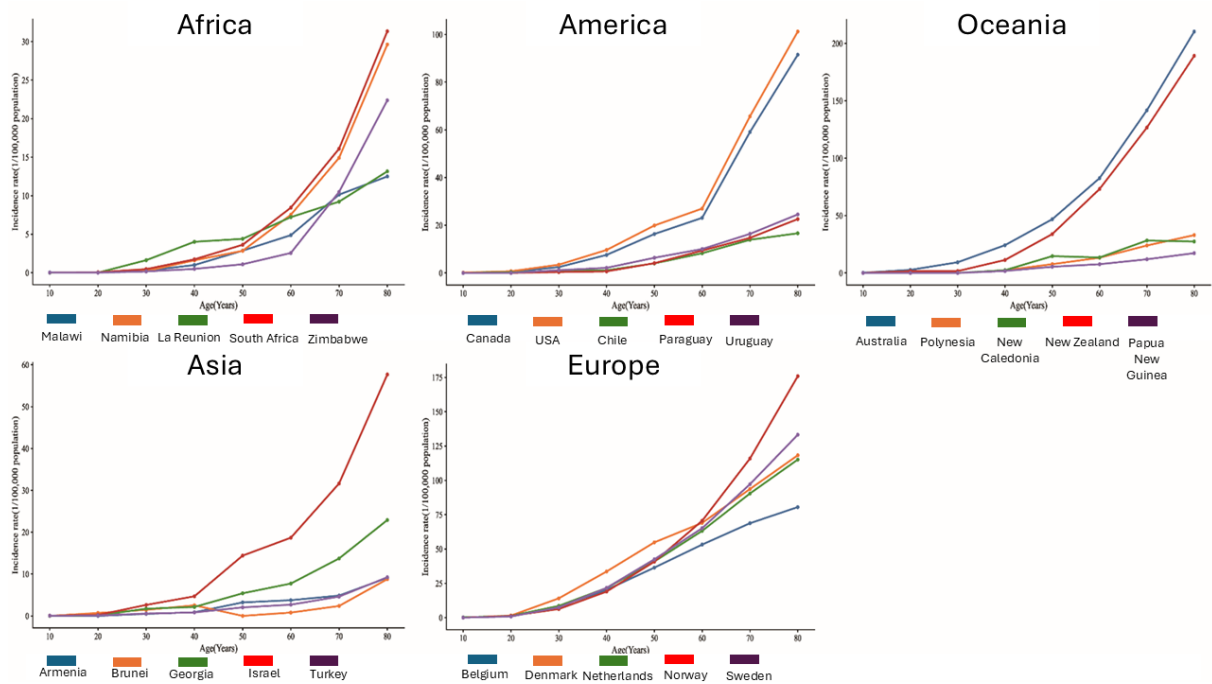
(a) Age-specific ASIR for NMSC across different countries by continent.



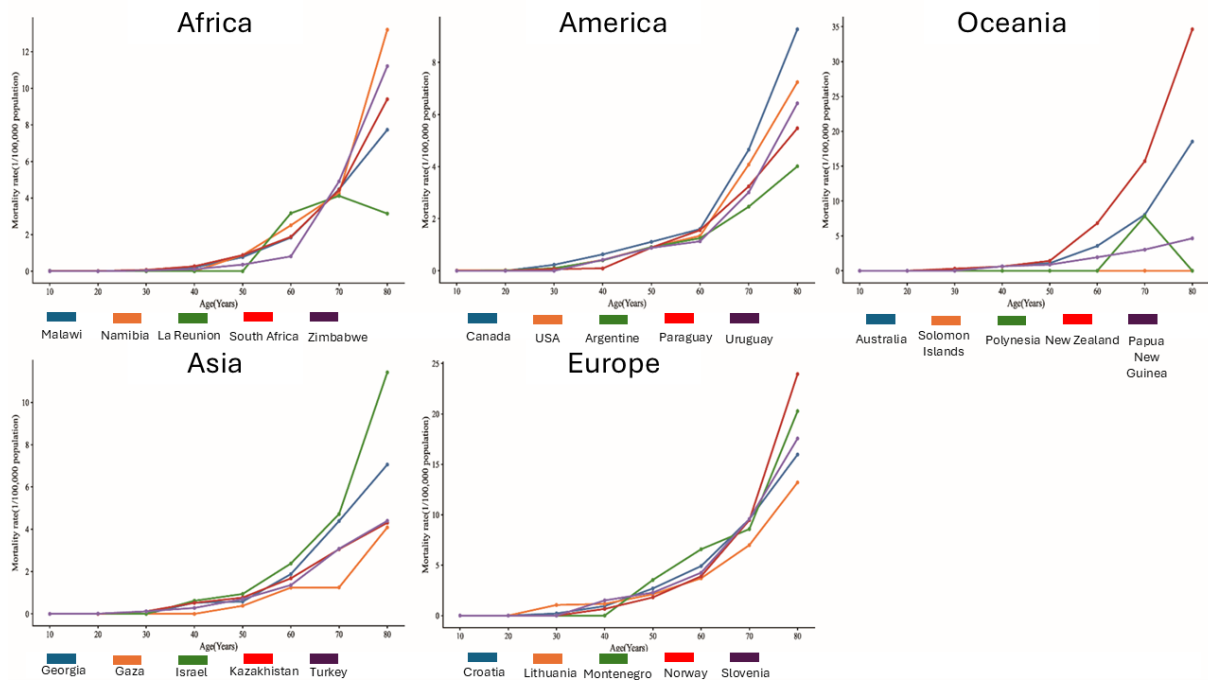
(b) Age-specific ASMR for NMSC across different countries by continent.

Figure 1.1.2: Age-specific incidence and mortality rates for NMSC across different countries grouped by continent. **Top:** ASIR. **Bottom:** ASMR. Adapted from [13]

melanoma ASIR values, with estimates exceeding 35 cases per 100,000 population, while several Northern European countries such as Denmark and Norway also show high ASIRs



(a) Age-specific ASIR for melanoma across different countries by continent.



(b) **Bottom:** Age-specific ASMR for melanoma across different countries by continent.

Figure 1.1.3: Age-specific incidence and mortality rates for melanoma across different countries grouped by continent. **Top:** ASIR. **Bottom:** ASMR. Adapted from [13]

(around 30–32 per 100,000), consistent with fair-skinned populations and behavioral exposure patterns. Conversely, many regions in Asia, the Middle East, and sub-Saharan Africa

report lower ASIRs—often below 10 per 100,000—partly due to higher melanin levels conferring photoprotection as well as differences in sun-exposure behavior [13]. However,

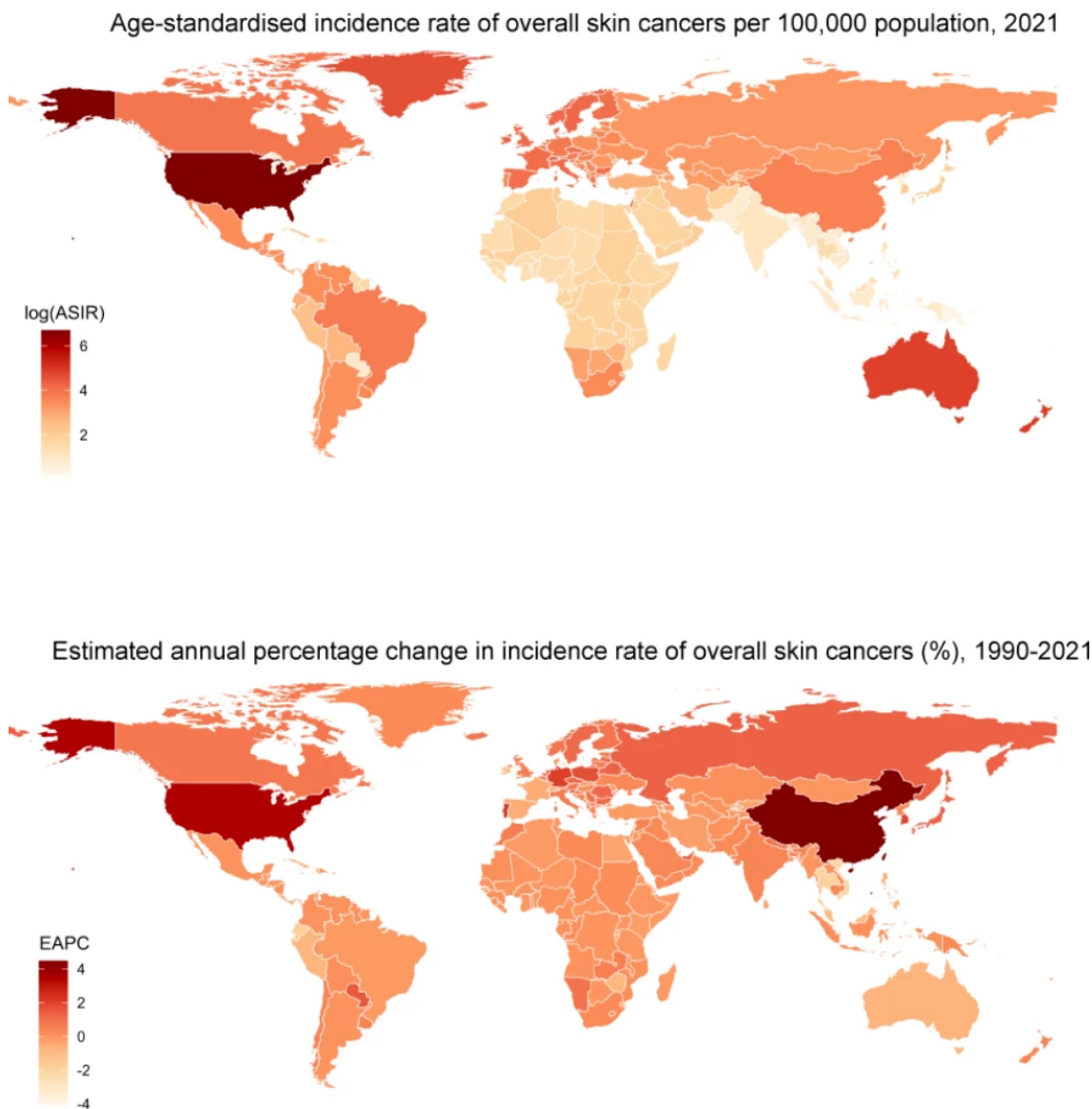


Figure 1.1.4: Global burden of overall skin cancers in 2021. **Top:** Log-transformed ASIR per 100,000 population in 2021. **Bottom:** Estimated annual percentage change (EAPC) in incidence rate (1990–2021). Adapted from [3].

these figures likely underestimate the true burden, since many low- and middle-income countries lack robust cancer registries and organized early-detection programs, leading to incomplete reporting and delayed diagnoses [16]. Moreover, socioeconomic stratifications show that high-SDI regions typically exhibit higher incidence but comparatively lower mortality, whereas middle- and low-SDI regions are experiencing rapidly increasing incidence trends, highlighting disparities in early detection and treatment [3].

1.2. Limitations of Current Diagnostic Techniques

During a dermatological examination, the doctor visually inspects the patient's skin using a dermoscope, which is nothing more than a magnifying lens equipped with a light source, to examine suspicious areas in greater detail.



Figure 1.2.1: Example of a handheld dermoscope used for magnified and illuminated inspection of skin lesions [17].

Dermoscopy enhances visualization of superficial structures located just below the skin surface, such as pigment networks, globules, vascular patterns, and other morphological features that are not visible to the naked eye [17]. By improving the visibility of cutaneous features, dermoscopy increases the sensitivity and specificity of the lesion's diagnosis compared to unaided inspection. Typical lateral resolution in dermoscopy is of the order of 10–20 μm , and its imaging depth is limited to the superficial epidermis, up to approximately 100 μm [18]. While dermoscopy significantly aids in distinguishing malignant from benign lesions compared to the naked eye, its diagnostic accuracy heavily relies on the doctor's experience and training. Representative dermoscopic patterns and their diagnostic descriptors used to support lesions classification are shown in Fig. 1.2.2. In less specialized clinics, diagnostic errors are more frequent, particularly for equivocal lesions where visual cues are subtle or ambiguous [19]. When lesions remain suspicious despite dermoscopic evaluation, in the vast majority of cases a biopsy is performed. This procedure involves surgically excising part or all of the lesion for histopathological examination, which remains the definitive method for confirming malignancy. Excised tissue is processed, sectioned, and stained (commonly with hematoxylin and eosin) to allow cellular-resolution assessment of architectural and cytological details [20]. In Fig. 1.2.3 a representative H&E-stained histopathology section of basal cell carcinoma is shown. Dermoscopy and histopathology differ fundamentally in the type of information they provide.

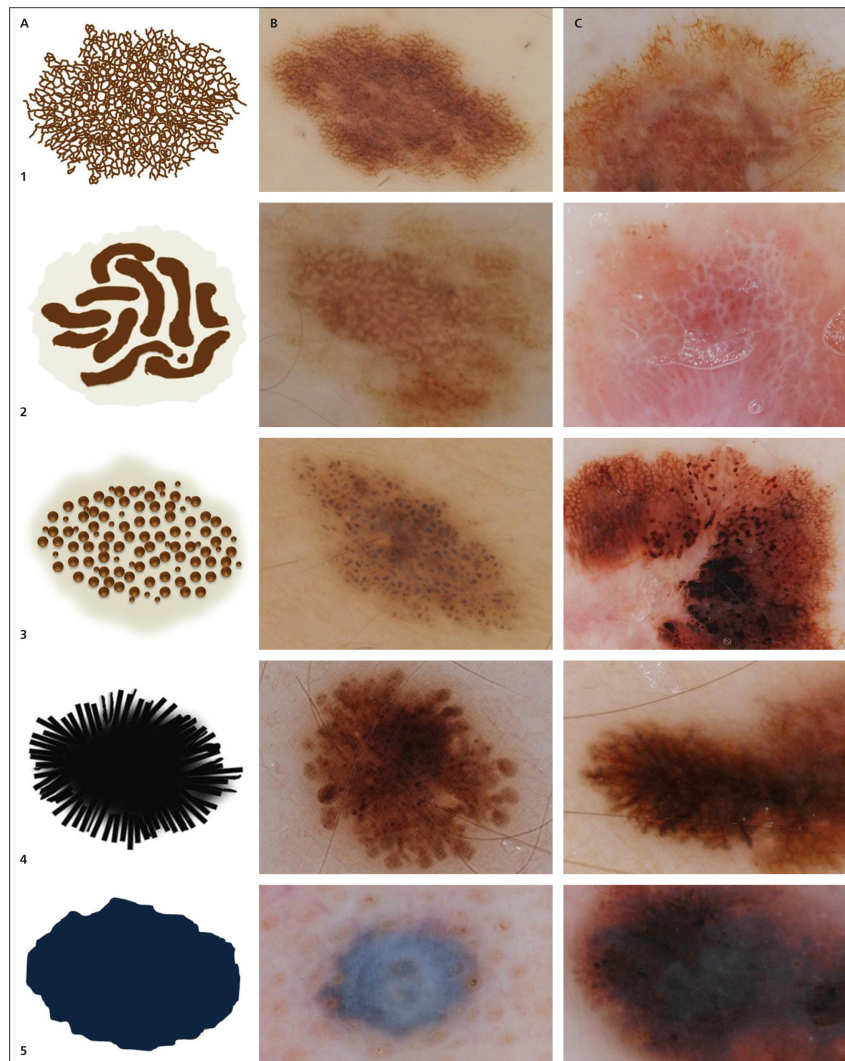


Figure 1.2.2: Examples of dermoscopic views and pattern descriptors used in clinical assessment [18, 19].

Dermoscopy gives a planar en face view of superficial structures, whereas histopathology provides cross-sectional images at cellular resolution. This difference is crucial for the diagnosis: as shown in Fig. 1.2.4, en face imaging (dermoscopy) and cross-sectional imaging (histopathology) provide complementary views of the epidermis and dermis structures that the dermatologist uses for the skin-lesion classification.

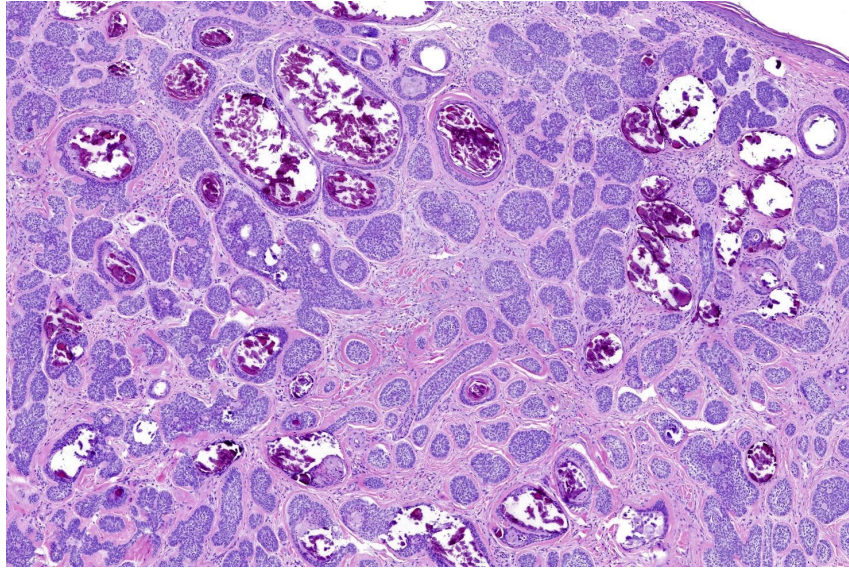


Figure 1.2.3: Example of an H&E-stained histopathology section (basal cell carcinoma), illustrating the cellular-level diagnostic information obtained after biopsy [20].

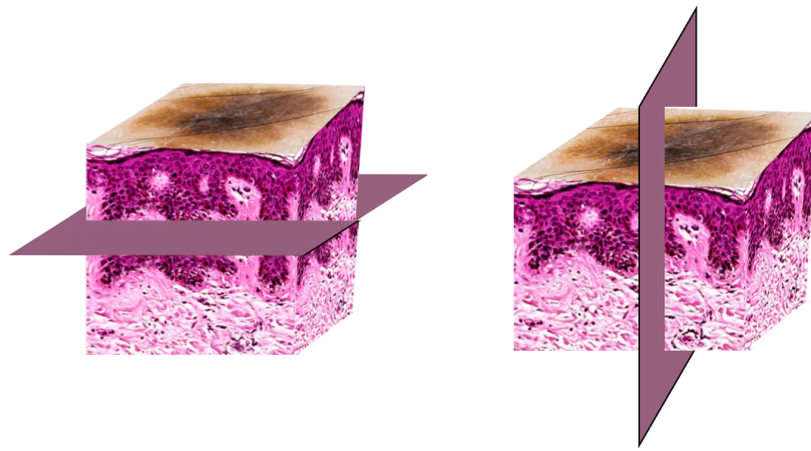


Figure 1.2.4: Conceptual difference between en face imaging (dermoscopy) and cross-sectional imaging (histopathology), highlighting complementary diagnostic information for skin-lesion classification [20, 21].

Dermoscopy presents intrinsic diagnostic limitations. It cannot reveal cellular morphology or cytologic atypia, which limits its specificity in ambiguous lesions. In addition, while dermoscopy is rapid and non-invasive, its shallow penetration restricts visualization of deeper dermal structures that may harbor malignant changes. False-positive diagnoses often lead to unnecessary excisions, while false-negative results may cause malignant lesions to be overlooked, with dangerous consequences for patients. Moreover, some malig-

nant tumors, such as amelanotic melanoma, spitzoid melanoma, and early in situ lesions, lack typical dermoscopic features, making them difficult to identify [22]. However, also biopsies have important limitations. They are invasive procedures that leave scars, may cause significant discomfort for patients, and involve healthcare costs related to surgical intervention, pathology analysis, and follow-up care [23]. Eventually, a substantial proportion of biopsies are found to be ultimately unnecessary: studies indicate that approximately 19 out of 20 biopsies in general dermatology clinics are performed on lesions that are benign, meaning that only about 1 in 20 is necessary to detect a melanoma [24]. Furthermore, a biopsy usually samples only a limited portion of the lesion, which may not capture the most representative area in heterogeneous tumors, potentially leading to under- or misdiagnosis in rare cases [25]. These factors collectively increase patient burden, raise healthcare costs, and highlight inefficiencies in current diagnostic workflows [26]. The limitations of dermoscopy and the high rate of unnecessary biopsies have motivated the development of non-invasive imaging methods such as reflectance confocal microscopy (RCM), a technique designed to bridge the gap between non-intrusive examination and surgical tissue removal. RCM enables in-vivo, non-invasive imaging of skin microstructure at cellular resolution, allowing assessment of both architectural and cytologic features without excision. By providing detailed visualization of the epidermis and superficial dermis (up to 300–400 μm), RCM can improve diagnostic specificity and reduce the number of unnecessary biopsies [27]. In summary, although dermoscopy has significantly improved clinical evaluation of suspicious skin lesions, its limitations in specificity, imaging depth, and reliance on the doctor’s expertise mean that invasive biopsies remain necessary in the majority of suspicious cases. The high proportion of unnecessary excisions, together with patient discomfort and healthcare costs, underscores the need for intermediate, high-resolution, non-invasive imaging modalities such as RCM.

1.3. Overview of Reflectance Confocal Microscopy History

Over the past few decades, reflectance confocal microscopy (RCM) has evolved from a proof-of-concept research tool to a technique that can be used in clinical instruments in dermatology [21]. The idea of performing in vivo, non-invasive optical sectioning of skin at cellular resolution was first explored in the 1990s, and the pioneering work by Rajadhyaksha et al. laid the foundation for modern RCM systems. Their early laboratory prototypes demonstrated that it was feasible to obtain real-time images of human skin in vivo using a scanning confocal configuration and intrinsic backscattering contrast, without the need for exogenous dyes [28]. In their landmark 1995 paper, “In vivo confocal scanning laser microscopy of human skin,” Rajadhyaksha and colleagues described the first video-rate confocal microscope for in vivo skin imaging. They systematically studied the influence of illumination wavelength on both image quality and penetration depth, comparing visible (400–700 nm) and near-infrared (NIR, 800–900 nm) ranges. Their results showed that visible light provides excellent contrast for epidermal structures, while NIR illumination allows visualization deeper into the papillary dermis, even revealing capillary blood cells. The authors eventually selected a wavelength of 830 nm as an optimal trade-off between deeper tissue penetration—limited primarily by scattering—and maintaining sufficient lateral resolution. They identified melanin as the main endogenous contrast agent, providing high signal levels particularly within pigmented structures such as basal keratinocytes and melanocytic lesions [28]. A representative reflectance confocal microscopy (RCM) image is shown in Fig. 1.3.1. In their 1999 follow-up article, Rajadhyaksha et al. presented significant advancements in instrumentation and an optimization of the key optical and mechanical parameters. They reported a measured lateral (in-plane) resolution of 0.5–1.0 μm and axial (optical sectioning) thickness of 3–5 μm using 830 nm illumination (and in some trials 1064 nm), achieving imaging depths up to approximately 350 μm over fields of view ranging from 160 μm x 160 μm to 800 μm x 800 μm . The study carefully investigated how the optical design—particularly the numerical aperture (NA) of the objective—and the scanning configuration influenced image quality and stability. By selecting a high-NA water-immersion objective (≈ 0.9 NA), they maximized lateral resolution while preserving sufficient optical sectioning capability. The use of a point-scanning raster pattern driven by galvanometric mirrors provided precise sampling, balancing frame rate, field of view, resolution and optical sectioning. The group also developed a custom mechanical stabilization device that was able to maintain skin motion within ± 25 μm , effectively mitigating motion artifacts during in vivo acquisition. To-

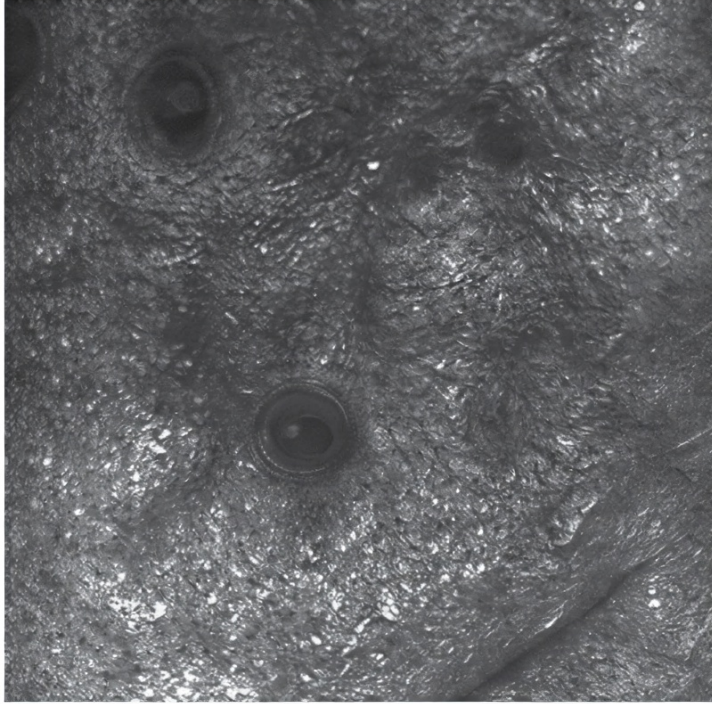


Figure 1.3.1: Example reflectance confocal microscopy (RCM) image showing skin microstructure in the en face plane [21].

gether, these optimizations defined a robust and repeatable design space—carefully tuned in NA, wavelength, and scanning geometry—that became the blueprint for all subsequent clinical RCM systems [29]. In the early 2000s, Rajadhyaksha and colleagues further refined RCM technology by exploring alternative scanning geometries. They investigated line-scanning and theta line-scanning configurations to improve imaging throughput or simplify the optical layout. However, point-scanning systems have remained the dominant configuration in clinical RCM instruments due to their superior sectioning ability and image contrast [30]. Throughout the 2000s and 2010s, Rajadhyaksha’s research continued to focus on enhancing RCM’s capabilities. Notably, in 2011, they published a study on the performance of full-pupil line-scanning reflectance confocal microscopy in human skin and oral mucosa in vivo, demonstrating the feasibility of this approach for high-resolution imaging in clinical settings [31]. Later, they introduced confocal mosaicking microscopy for rapid imaging of large areas of excised tissue, facilitating real-time surgical pathology [32, 33]. Mosaics are formed by stitching RCM images to extend the field of view beyond a single frame, as illustrated in Fig. 1.3.2.

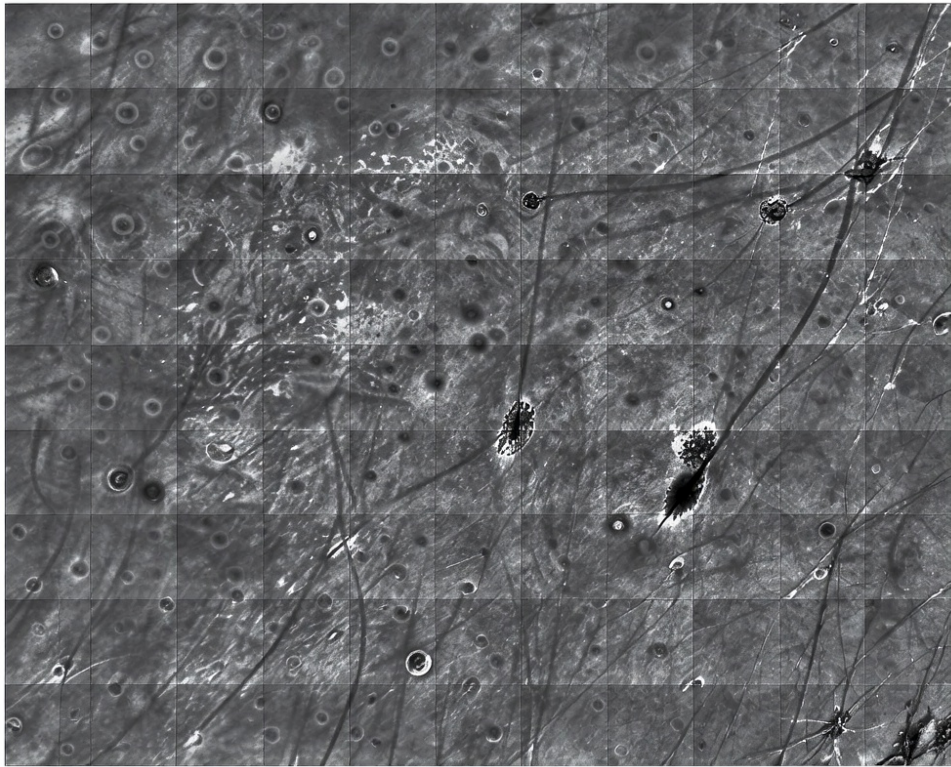


Figure 1.3.2: Example of RCM mosaicking: 9 x 11 adjacent confocal frames stitched to increase the effective field of view of 0.5 mm x 0.5 mm while preserving cellular detail to 4.5 mm x 5.5 mm [32].

All these works laid the foundations for the development of the first clinical RCM devices, which were subsequently optimized through multiple translation-to-clinic efforts into the modern instruments currently used in hospitals.

1.4. Reflectance Confocal Microscopy Current State of the Art

These laboratory prototypes were eventually translated into commercial instruments by Lucid Inc. (later acquired by Caliber I.D., now Caliber Imaging & Diagnostics), which introduced the VivaScope product line and remains the leading company in clinical RCM technology [34]. Their principal clinical systems include the VivaScope 1500, a stationary benchtop system, and the VivaScope 3000, a handheld probe [35]. According to manufacturer specifications, the VivaScope 1500 (Fig. 1.4.1) employs 830 nm laser illumination, achieving horizontal (lateral) resolution $< 1.25 \mu\text{m}$ and vertical (axial) optical sectioning $< 5.0 \mu\text{m}$, with frame rates ≥ 9 fps over a $500 \mu\text{m} \times 500 \mu\text{m}$ field of view. The system supports mosaic imaging over regions up to 8×8 mm, combining a high-NA (≈ 0.9 NA) water-immersion objective with a galvanometer-based point-scanning mechanism. Independent evaluations have confirmed these specifications in clinical settings, reporting lateral resolution around $1 \mu\text{m}$ and axial sectioning between $3\text{--}5 \mu\text{m}$ [36].



Figure 1.4.1: The VivaScope 1500 wide-probe reflectance confocal microscope, commonly used for in vivo skin imaging in clinical settings [35, 37].

The VivaScope 3000, introduced more recently, adopts a compact handheld probe configuration that enables direct scanning on curved or difficult-to-reach skin areas. While offering greater flexibility and ergonomic usability, it typically sacrifices some field-of-view and mosaicking capability compared to the benchtop system (Fig. 1.4.2). Despite these differences, the core optical design—830 nm illumination, high-NA objective, and point-scanning confocal architecture—has remained essentially unchanged since the first



Figure 1.4.2: The VivaScope 3000 handheld reflectance confocal microscope, designed for imaging curved and hard-to-access anatomical sites [37].

prototypes [37]. This enduring design consistency likely reflects both the robustness of the original engineering choices and the company’s dominant market position. However, one of the major impediments to the widespread adoption of RCM remains its extremely high cost. Independent health-technology assessments have reported acquisition and maintenance expenses exceeding 100,000 USD. In addition to the purchase price, this device requires trained operators, periodic calibration, and proprietary software licenses—all contributing to a substantial total cost [38, 39]. Consequently, these devices are found almost exclusively in well-funded dermatology centers or research hospitals in wealthier countries. Smaller clinics and resource-limited regions rarely have access to such equipment, meaning that even in developed nations, patients must often travel to specialized hospitals in major cities to benefit from RCM imaging. Recent studies have focused on the integration of artificial intelligence (AI) with reflectance confocal microscopy to enhance diagnostic accuracy and reduce operator dependency. Machine learning algorithms have been developed to automatically identify and classify cellular and architectural patterns associated with melanoma, basal cell carcinoma, and other skin lesions directly from RCM images. Early results indicate that AI-assisted RCM can achieve diagnostic performance comparable to expert dermatologists, potentially enabling non-specialist clinicians to utilize RCM effectively [40, 41]. This approach also allows for faster image interpretation and standardized reporting, paving the way for broader clinical adoption of RCM in dermatology. Another significant advancement in clinical RCM practice is the implementation of video-mosaicking, a technique that enables the real-time acquisition of consecutive confocal images which are then digitally stitched together to create a high-resolution mosaic covering a larger skin area of an arbitrary shape, as displayed in Fig. 1.4.3. This approach

overcomes the inherently limited field of view of point-scanning RCM systems, allowing dermatologists to examine extensive lesions while preserving cellular-level detail.

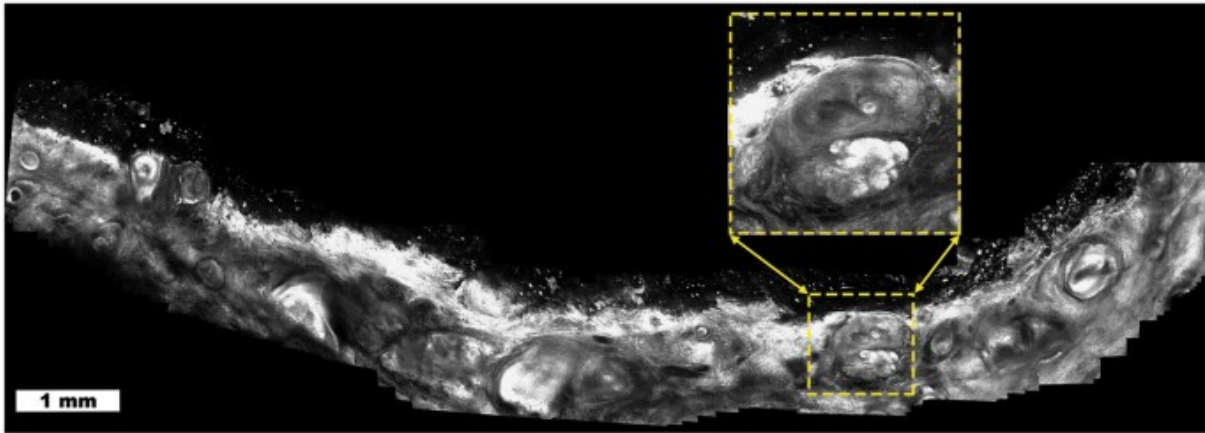


Figure 1.4.3: Example of video mosaicking in reflectance confocal microscopy: consecutive frames are registered and stitched into a large-area mosaic of arbitrary shape while preserving cellular detail [42, 43].

Video-mosaicking requires precise synchronization between scanning speed, frame acquisition, and software-based image registration to ensure seamless stitching and minimal motion artifacts. Clinical studies have demonstrated that this method improves diagnostic confidence, facilitates lesion mapping, and enables more accurate correlation with histopathology, particularly for heterogeneous or large lesions. The integration of video mosaicking with RCM has thus become a key feature for bridging the gap between localized cellular imaging and clinically relevant assessment of entire lesions [42–44]. In summary, today’s RCM instruments still carry the core design principles established by Rajadhyaksha’s prototypes: high-NA confocal optics, near-infrared illumination, and point-scanning architecture. Commercial systems such as the VivaScope 1500 and VivaScope 3000 deliver histology-like en face images with cellular resolution and large-area mosaicking capabilities, but their prohibitive cost and complexity restrict their accessibility. These limitations have motivated recent efforts toward simplified, lower-cost RCM architectures aiming to preserve image quality while improving affordability and usability.

1.5. Goals and Structure of this Thesis

This thesis presents the design, integration, and experimental validation of the illumination and scanning backbone of a compact reflectance confocal microscope (RCM) prototype. Rather than delivering a fully operational clinical RCM system, the work focuses on the most timing-critical, opto-mechanically sensitive, and software-intensive subsystems, namely the illumination path, high-speed raster scanning architecture, synchronization electronics, and image reconstruction pipeline. The confocal detection arm, although included in the conceptual system architecture and analyzed at the theoretical level, was not aligned and experimentally validated within the time frame of this thesis, in order to concentrate efforts on establishing a robust and well-characterized scanning and acquisition platform. Prior to prototype implementation, the thesis analyzes the physical principles and engineering trade-offs underlying reflectance confocal microscopy, with emphasis on confocal optical sectioning, contrast formation in backscattering media, and the dependence of lateral and axial resolution on numerical aperture, wavelength, and pinhole size. This theoretical framework defines the performance targets of the final intended system and guides the optical and mechanical design choices adopted for the illumination arm and scanning subsystem. The primary experimental objective of this work is the realization and validation of a high-speed raster scanning module based on a rotating polygon mirror for fast-axis deflection and a galvanometric scanner for slow-axis deflection, combined with two properly conjugated $4f$ relay systems. Particular attention is devoted to preserving pivot conjugation, minimizing beam clipping at large deflection angles, and ensuring stable telecentric propagation toward the objective back focal plane. A key requirement is achieving video-rate operation on the order of 10 frames per second, which necessitates careful co-design of scanner drive electronics, detector bandwidth, analog front-end conditioning, digitization, and software-based frame reconstruction. The work therefore places strong emphasis on temporal synchronization between scanning elements and data acquisition, as well as on the development of a flexible reconstruction algorithm capable of compensating for non-ideal scan duty cycles and geometric distortions. An additional objective is the implementation of an axial translation capability compatible with the final confocal architecture, enabling depth scanning over several hundred micrometers. Although volumetric confocal imaging is not demonstrated experimentally in the present prototype, the mechanical and optical provisions required for such functionality are incorporated into the system design. From a practical standpoint, the thesis encompasses the complete realization of the illumination and scanning platform. This includes analytical optical design, selection of commercial components, mechanical integration, fabrication of custom mounts, electronic interfacing of scanners and detector,

development of control firmware and acquisition software, and full system assembly and alignment. Experimental validation is performed in transmission mode using calibrated resolution targets and biological samples. These measurements allow quantitative assessment of raster synchronization fidelity, lateral resolving capability in the image plane of the second relay system, contrast performance, and overall stability of the temporal-to-spatial mapping chain. Following this introduction, the thesis is organized as follows. Chapter 2 reviews the physical foundations of reflectance confocal microscopy and defines the theoretical performance limits of the intended system. Chapter 3 presents the optical and mechanical design of the illumination and scanning architecture. Chapter 4 describes the electronics, synchronization strategy, and image reconstruction algorithms. Chapter 5 reports the assembly, alignment, and experimental validation of the raster scanning subsystem. Chapter 6 presents imaging results on biological samples obtained in transmission configuration, demonstrating the practical functionality of the developed platform. Finally, Chapter 7 summarizes the main contributions of the work and outlines the steps required to complete the reflectance confocal implementation and move toward a compact, cost-effective RCM instrument.

2 | Principles of Reflectance Confocal Microscopy

2.1. Fundamentals of Confocal Imaging

A typical reflectance confocal microscope for biological applications employs a continuous-wave (CW), single-mode laser as its illumination source, most commonly in the near-infrared spectral range around 830 nm [45]. As mentioned in the previous chapter, this wavelength represents an optimal balance between a clinically adequate lateral resolution and a decent penetration depth in biological tissues [46]. The scheme of a typical RCM setup is shown in Fig. 2.1.1.

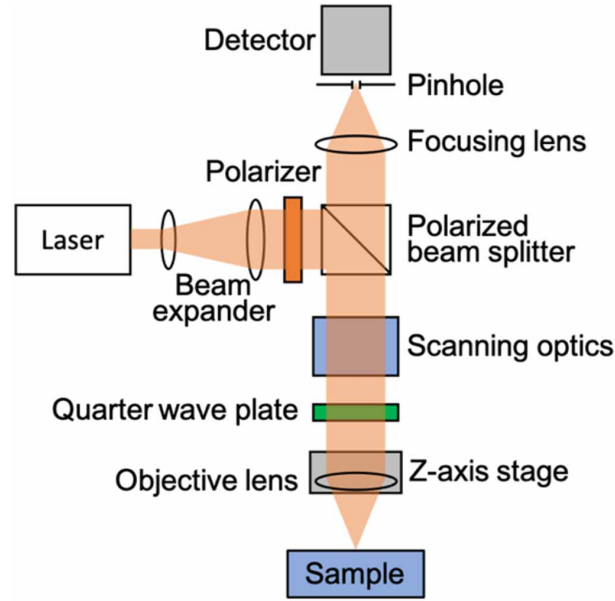


Figure 2.1.1: Schematic diagram of a reflectance confocal microscopy (RCM) system, highlighting the main optical components and the detection path [47].

The laser beam is first conditioned through a set of optics designed to ensure high spatial quality and proper beam diameter. This conditioning stage usually includes a beam expander or telescope to adjust the beam size, an optical isolator to prevent back-reflections into the laser cavity, and a spatial filter to remove higher-order transverse modes. Moreover, a polarizer defines a precise linear polarization state before the beam enters the main optical path. The linearly polarized laser beam then encounters a polarizing beam splitter, oriented to transmit one polarization (for instance, p-polarized light) toward the scanning system while reflecting the orthogonal polarization (s-polarized light) into a beam dump [48]. After passing through the PBS, the beam reaches the scanning subsystem. Scanning is often achieved by two galvanometric mirrors, which are small, high-precision mirrors mounted on rapidly rotating motors that can deflect the beam along two orthogonal directions. The first mirror, typically referred to as the fast scanner, produces the horizontal scan, while the second mirror, the slow scanner, incrementally steps the beam vertically

to obtain a two-dimensional raster pattern, as shown in Fig. 2.1.2.

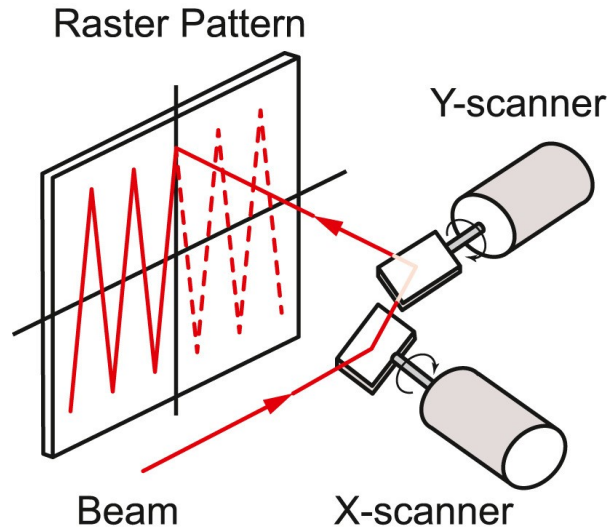


Figure 2.1.2: Raster scan pattern produced by a pair of galvanometric mirrors for two-dimensional beam steering in reflectance confocal microscopy [49].

These galvanometric mirrors are positioned in such a way that they are optically conjugated with the back focal plane (BFP) – or pupil plane – of the microscope objective. This conjugation ensures that angular deflections at the scanners correspond to lateral displacements of the focal spot on the sample without introducing vignetting or beam walk across the pupil. The scanning system is usually relayed by a pair of lenses arranged in a 4-f configuration (a scan lens and a tube lens), which image the scan plane onto the objective's back aperture and maintain the telecentricity of the beam, as represented in Fig. 2.1.3.

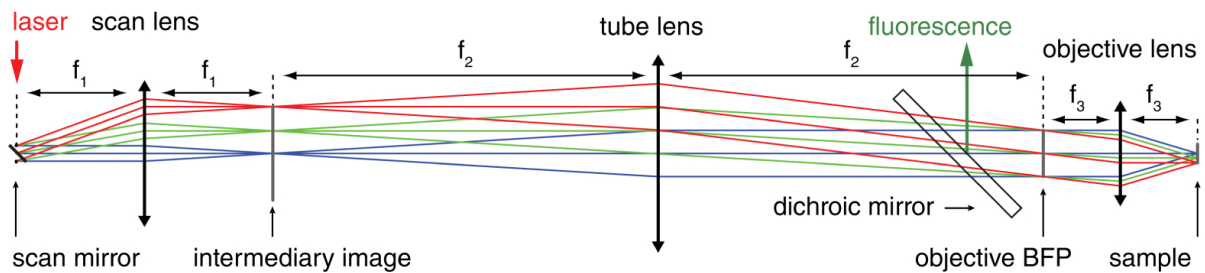


Figure 2.1.3: Optical relay scheme showing the scan lens and tube lens configuration used to image the scanning mirrors onto the objective pupil while maintaining telecentric scanning at the sample [50].

This arrangement ensures that the numerical aperture and illumination conditions remain constant across the entire field of view, preserving uniform resolution and brightness [51]. Just before entering in the objective lens, the beam passes through a quarter-

wave plate whose fast axis is oriented at 45° with respect to the incoming polarization. This optical element converts the linearly polarized light into circularly polarized light - Fig. 2.1.4 -, which is the polarization state that the light keeps for the remaining part of the illumination path and inside the sample. Upon reflection and backscattering from the sample structures, the light retraces its path through the same optical elements and, during this return trip, the circularly polarized light passes again through the quarter-wave plate, which now converts it back into linearly polarized light, rotated by 90° with respect to the original illumination polarization direction.

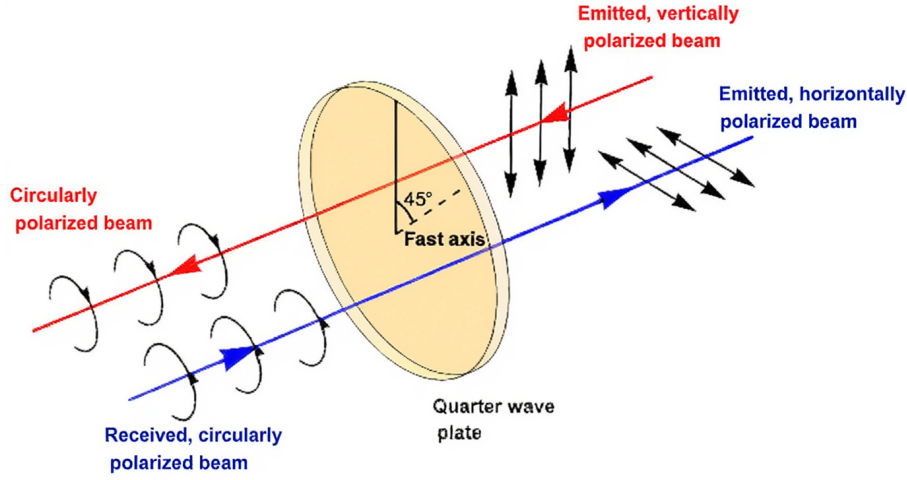


Figure 2.1.4: Operating principle of a quarter-wave plate (QWP): it introduces a $\pi/2$ phase delay after the single passage of light polarized linearly with polarization direction forming a $\pi/4$ angle with the fast and slow axes [52].

As a consequence, when the returning beam reaches the PBS, it is directed toward the detection arm rather than back toward the laser source. Fig. 2.1.5 shows the PBS working principle: this polarization-based separation effectively decouples the illumination and detection paths while maintaining a compact epi-illumination geometry.

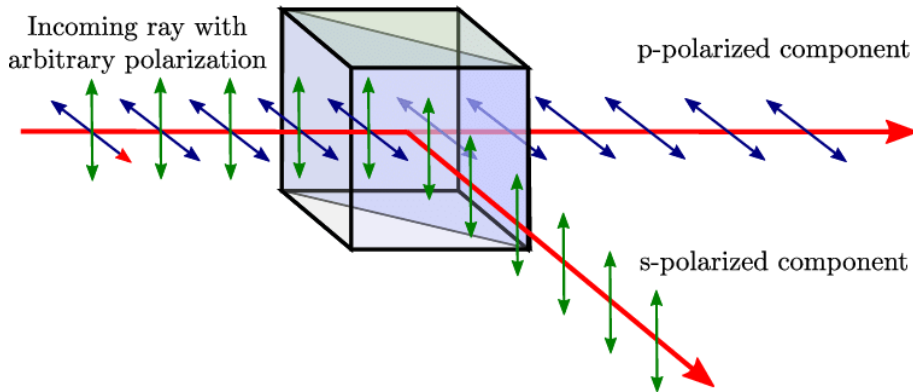


Figure 2.1.5: Operating principle of a polarizing beam splitter (PBS): it separates an incident beam into transmitted and reflected components based on the light polarization state, enabling polarization-based routing of the illumination and backscattered light [53].

The objective lens focuses the scanned beam into the sample, where it illuminates a single diffraction-limited spot. As the galvanometric mirrors deflect the beam, the focus scans sequentially across the sample, point by point, forming the basis for pixel-wise image acquisition, as represented in Fig. 2.1.6.

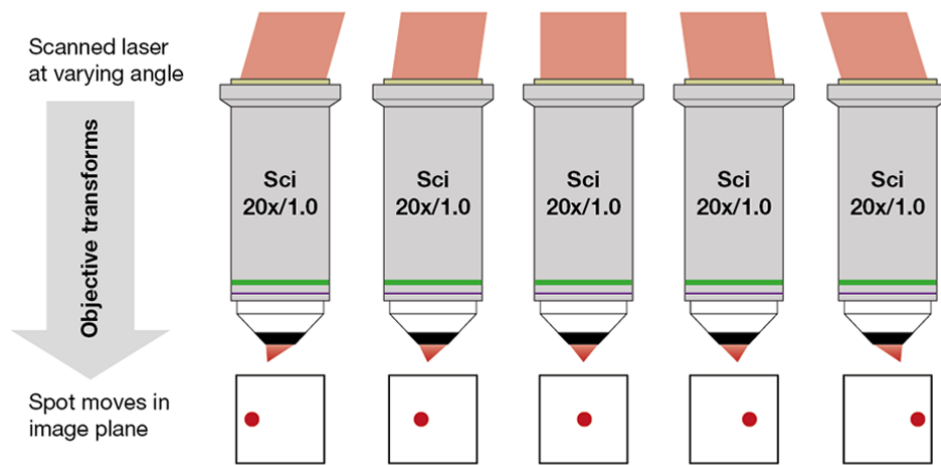


Figure 2.1.6: Schematic representation of how the microscope objective focuses the illumination into a diffraction-limited spot scanning on the sample in correspondence of different beam feed angles [54].

The light backscattered from each illuminated point in the sample is collected by the same objective and retraces the illumination path in reverse. When it encounters the scanning mirrors, their instantaneous deflection angles cause the returning beam to be descanned, meaning that the beam motion induced by the scanners is effectively cancelled out. This descanning process ensures that the backscattered light from all scan positions is reimaged onto a fixed location in the detection plane. Consequently, a stationary confocal pinhole is used to spatially filter the light, while a single fixed detector collects the signal. The descanning mechanism is fundamental for a stable image reconstruction, as it guarantees that the confocal aperture and detector remain optically aligned with the instantaneous focal position during the entire scan. In the detection arm, the returning light passes through an imaging system that focuses it onto the confocal aperture. This aperture, or pinhole, is placed in a plane conjugate to the focal plane of the objective. Its function is to reject light originating from out-of-focus planes. Light scattered from regions outside the focal plane forms a defocused spot at the pinhole plane and is therefore largely blocked,

while light from the focal plane passes through the aperture and reaches the detector. This spatial filtering is what gives the confocal microscope its axial sectioning capability, allowing it to produce thin optical slices of the specimen. This concept of confocality is illustrated in Fig. 2.1.7.

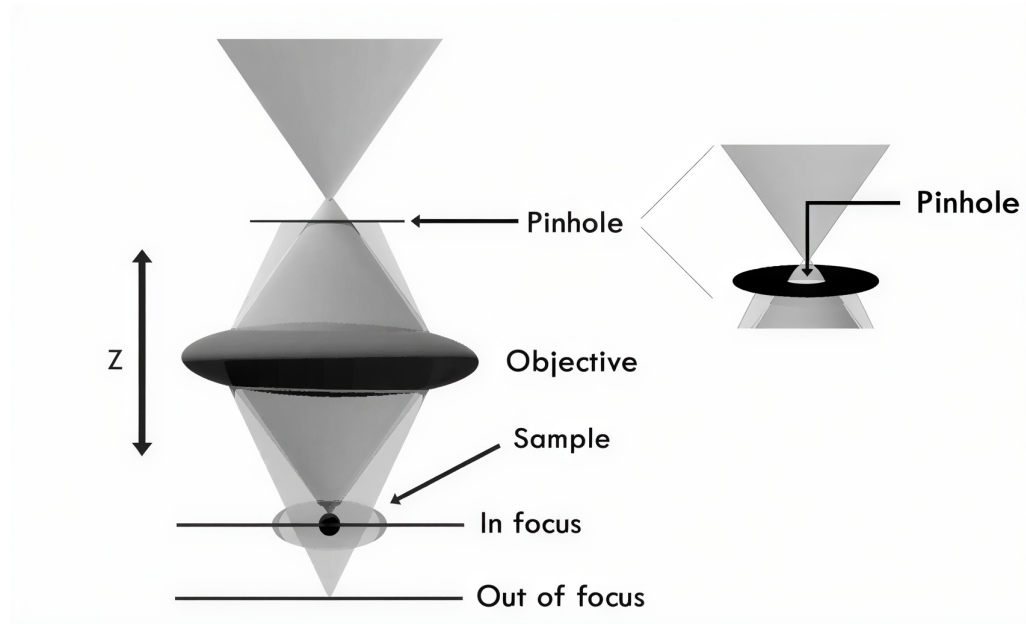


Figure 2.1.7: Illustration of the confocal principle: spatial filtering by a pinhole suppresses out-of-focus light, improving contrast and enabling optical sectioning [55].

The effect of the pinhole size will be discussed in detail in a dedicated section since it plays a crucial role: if the pinhole is too small, signal intensity and signal-to-noise ratio decrease, while if it is too large, optical sectioning is degraded. In many modern instruments, a single-mode optical fiber is used in place of a physical pinhole, serving both as the spatial filter and as a flexible light guide for routing the signal to the detector. The transmitted light after the pinhole is detected by a point detector, such as an avalanche photodiode (APD) or a photomultiplier tube (PMT), capable of measuring low-intensity optical signals with high bandwidth. The detector converts the optical intensity into an electrical signal that is synchronized with the position of the galvanometric mirrors. The scanning control electronics generate timing signals—such as line synchronization and frame triggers—that are used by the acquisition system to map each intensity measurement to its corresponding (x, y) coordinate on the sample. This process reconstructs the two-dimensional image from the sequential point-by-point measurements. Because RCM uses a continuous-wave laser, the detection system integrates the backscattered intensity within the dwell time of each pixel, typically on the order of microseconds. The frame rate of the system depends on several factors, such as the scanners' oscillation frequencies, the number of pixels per frame and the detector bandwidth. Precise synchro-

nization among the scanning system, data acquisition, and image reconstruction pipeline is crucial to ensure spatial accuracy and to prevent image distortions, particularly at high scanning speeds. Another crucial aspect is the choice of the objective and immersion medium, which critically influences the image quality in RCM. A high-NA objective (typically $NA \gtrsim 0.8\text{--}1.0$) maximizes both lateral and axial resolution and boosts collection efficiency in epi-detection, since a larger solid angle is sampled. Even though it will be explained further in a dedicated section, at first order the confocal lateral resolution scales as

$$\Delta x \approx 0.4 \frac{\lambda}{NA}, \quad (2.1)$$

while the axial sectioning scales as

$$\Delta z \approx 1.4 n \frac{\lambda}{NA^2}, \quad (2.2)$$

(for a small pinhole), underscoring the need for high NA to achieve thin optical slices and cellular-level resolution. Equally important is the refractive-index matching at the specimen interface. In vivo skin RCM commonly employs water-immersion objectives and index-matching water-based gels with $n \approx 1.33$ to reduce Fresnel reflections at the window/skin boundary and to mitigate spherical aberration introduced by the stratified indices of the epidermis' layers. In summary, a standard reflectance confocal microscope operates by directing a continuous-wave, single-mode laser beam through beam-conditioning optics, a polarization-based beam splitter, and a pair of galvanometric mirrors that scan the beam across the sample through a high numerical aperture objective. The backscattered light is descanned by the same mirrors, separated from the illumination path by polarization rotation operated by a quarter wave plate, spatially filtered through a confocal pinhole or optical fiber, and finally detected by a sensitive photodetector synchronized with the scanning system. This configuration provides all the capabilities of confocal microscopy: optical sectioning, high contrast, and cellular-level resolution in real time [56].

2.2. Lateral Resolution in Reflectance Confocal Microscopy

In general, the lateral resolution is the minimum centre-to-centre spacing between two object points in the focal plane that can still be discriminated as separate, and it is caused by the diffraction at the finite pupil of the objective. For a circular aberration-free pupil, the image of a point-like source is the well known Airy disk. The objective lens can be modelled by a uniform circular pupil $P(\rho)$ defined as:

$$P(\rho) = \begin{cases} 1, & 0 \leq \rho \leq 1, \\ 0, & \text{otherwise.} \end{cases} \quad (2.3)$$

Where ρ is the normalized pupil radius. In the Debye-Fraunhofer picture, the focal-plane field is (up to a global phase term) the 2-D spatial Fourier transform of the pupil. With cylindrical symmetry, it is convenient to adopt polar coordinates in both pupil (ρ, φ) and image (r, ψ) planes. The contribution to the phase term of an image point at (r, ψ) from a pupil point located at (ρ, φ) is:

$$e^{i\gamma\rho\cos(\varphi-\psi)}. \quad (2.4)$$

Where the dimensionless spatial frequency γ is defined as:

$$\gamma \equiv \frac{2\pi}{\lambda_0} NA r, \quad (2.5)$$

where $NA = n \sin(\theta_{\max})$ is the object-space numerical aperture of the objective lens – i.e. the NA of the focused cone at the sample – in a medium of refractive index n , and λ_0 is the vacuum wavelength. The on-axis normalized amplitude point-spread function is therefore:

$$A(r) \propto \iint_{\rho \leq 1} e^{i\gamma\rho\cos(\varphi-\psi)} \rho d\rho d\varphi. \quad (2.6)$$

The azimuthal integral is a standard identity,

$$\int_0^{2\pi} e^{i\gamma\rho\cos(\varphi-\psi)} d\varphi = 2\pi J_0(\gamma\rho), \quad (2.7)$$

which reduces the problem to a single radial Hankel transform:

$$A(r) \propto 2\pi \int_0^1 J_0(\gamma\rho) \rho d\rho. \quad (2.8)$$

Using the known Bessel integral

$$\int_0^1 \rho J_0(\gamma \rho) d\rho = \frac{J_1(\gamma)}{\gamma}, \quad (2.9)$$

we obtain the normalized Airy amplitude

$$\frac{A(r)}{A(0)} = \frac{2J_1(\gamma)}{\gamma}. \quad (2.10)$$

The intensity point-spread function is then

$$I(r) = I_0 \left[\frac{2J_1(\gamma)}{\gamma} \right]^2 \quad (2.11)$$

where J_1 is the first-order Bessel function, represented in Fig.. The first zero of the Airy

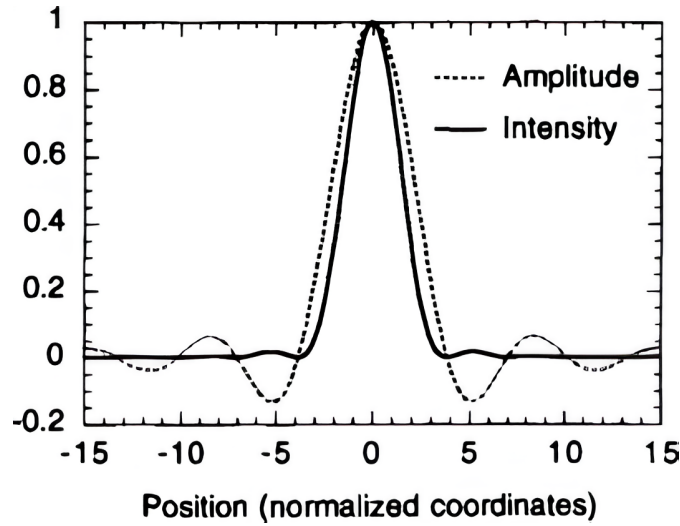


Figure 2.2.1: Illustration of the point-spread function (PSF) of an imaging system, showing how a point-like object is mapped into an extended intensity distribution, which sets a finite and non-ideal lateral resolution [57].

disk occurs at

$$r_{\text{Airy}} = \frac{1.22 \lambda_0}{2 NA} = 0.61 \frac{\lambda_0}{NA}, \quad (2.12)$$

which is the classical Rayleigh separation for two equal-intensity point images in widefield microscopy. In a confocal system, a pinhole placed in a position conjugated to the focal plane rejects light that is not imaged to its aperture. Under linear detection in the small-pinhole regime, the detected signal from an object point is proportional to the product of the illumination intensity at that point and the collection efficiency of the imaging path.

Accordingly, the intensity point-spread function factorizes as:

$$\text{PSF}_{\text{confocal}}(x, y, z) = \text{PSF}_{\text{illum}}(x, y, z) \text{PSF}_{\text{detect}}(x, y, z), \quad (2.13)$$

Meaning that only the same object location that is both illuminated and imaged onto the pinhole contributes to the measured signal. Therefore, one obtains the following result:

$$\text{PSF}_{\text{confocal}}(r) \propto \left[\frac{2J_1(\gamma)}{\gamma} \right]^4 \quad (2.14)$$

Which is the square of the widefield intensity PSF, which narrows the main lobe and strongly suppresses sidelobes [58, 59].

For closed-form solutions near focus, it is convenient to approximate the central Airy lobe with a rotationally symmetric Gaussian intensity [60]:

$$I(r) = e^{-2(r/w_0)^2}, \quad (2.15)$$

Where w_0 is the $1/e^2$ beam-waist radius in the focal plane. The intensity point-spread functions of illumination and detection are then modelled as:

$$\text{PSF}_{\text{illum}}(r) = e^{-2(r/w_0)^2}, \quad \text{PSF}_{\text{detect}}(r) = e^{-2(r/w_0)^2}. \quad (2.16)$$

Mapping the Airy central lobe to a Gaussian can be done by curvature, FWHM or $1/e^2$ matching: all consistent choices that yield the same λ/NA dependence, but slightly different numerical pre-factors. For example, using the widely adopted FWHM-matching criterion, would lead to the following expression for w_0 :

$$w_0 \approx 0.437 \frac{\lambda_0}{NA}. \quad (2.17)$$

The FWHM of the widefield intensity Airy disk function

$$\left[\frac{2J_1(\gamma)}{\gamma} \right]^2$$

Is the well-known Abbe limit:

$$\Delta x_{\text{FWHM,wide}} \simeq 0.514 \frac{\lambda_0}{NA}, \quad (2.18)$$

while a Gaussian curve of the form $e^{-2(r/w_0)^2}$ has known FWHM given by

$$\text{FWHM} = w_0 \sqrt{2 \ln 2}. \quad (2.19)$$

Because the illumination and detection Gaussian intensity point-spread functions multiply, the confocal intensity point-spread function remains a Gaussian in the following form: $e^{-4(r/w_0)^2}$, with half the width due to the squaring operation. Therefore,

$$\Delta x_{\text{FWHM,conf}} = \frac{\Delta x_{\text{FWHM,wide}}}{\sqrt{2}} \approx 0.364 \frac{\lambda_0}{NA}, \quad (2.20)$$

Which is in close agreement with the non-Gaussian evaluation of the exact width of $[2J_1(\gamma)/\gamma]^4$, which gives

$$\Delta x_{\text{FWHM,conf}} \approx 0.369 \frac{\lambda_0}{NA}. \quad (2.21)$$

If one prefers to use the Rayleigh metric, which states that two equal Airy patterns are “just resolved” when the peak of one coincides with the first zero of the other (Fig. 2.2.2 and Fig. 2.2.3), then he should consider

$$\Delta x_{\text{FWHM,wide}} \simeq 0.61 \frac{\lambda_0}{NA}. \quad (2.22)$$

In Fig. 2.2.2 the more relaxed Sparrow criterion is also shown. This criterion states that two points are just resolved when the central dip in the combined intensity profile disappears, creating a flat-topped plateau. For the confocal point-spread function $\propto [2J_1(\gamma)/\gamma]^4$ the zero locations are unchanged under the squaring operation and this leads to the same result. However, what matters for practical discrimination is the contrast at the saddle between two near peaks. Because the confocal PSF is narrower and has much weaker sidelobes, the central dip becomes sufficiently deep at smaller spacings than in widefield. A convenient Rayleigh-style adaptation is therefore to rescale the widefield Rayleigh separation by the same factor $\sqrt{2}$ that governs the PSF width (from the Gaussian or FWHM analysis) [56]. This yields

$$\Delta x_{\text{Rayleigh,conf}} \approx \frac{0.61}{\sqrt{2}} \frac{\lambda_0}{NA} \approx 0.431 \frac{\lambda_0}{NA}. \quad (2.23)$$

In practice, the $\sqrt{2}$ gain holds for small pinholes (roughly $\lesssim 0.5$ –1 Airy unit). As the pinhole is enlarged the PSF gradually reverts toward the widefield limit ($\Delta x \rightarrow 0.514 \lambda_0/NA$) while the detected signal increases. The squaring of the PSF also suppresses sidelobes: the first Airy ring, about 1.7% of the peak in intensity, is reduced to $\sim 0.03\%$ in confocal detection, improving image contrast. To keep a single, practical figure for estimates and

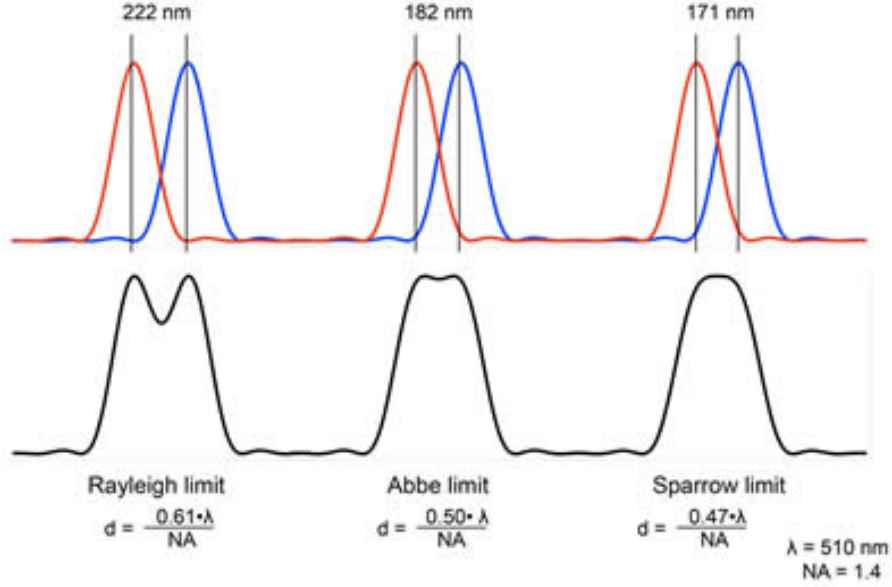


Figure 2.2.2: Illustration of different lateral resolution criteria [61].

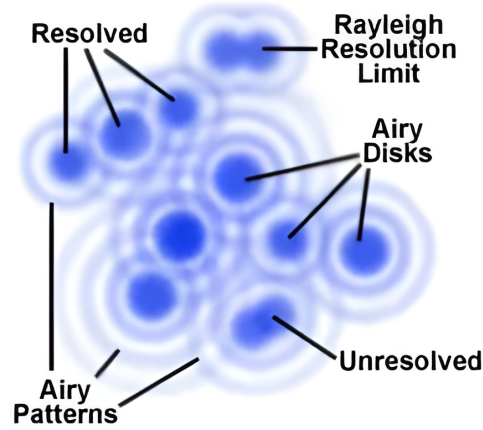


Figure 2.2.3: Illustration of lateral resolution concept in confocal microscopy, showing the minimum resolvable separation between two adjacent features [62].

comparisons, it is widely adopted to quote the lateral resolution as

$$\Delta x \approx 0.4 \frac{\lambda_0}{NA} \quad (2.24)$$

for reflection confocal imaging with a small pinhole under aberration-free, diffraction-limited conditions. This choice sits between the FWHM-based $0.364 \lambda_0 / NA$ and the Rayleigh-style $0.431 \lambda_0 / NA$, and provides a compact, conservative rule-of-thumb [58, 59].

2.3. Axial Resolution in Reflectance Confocal Microscopy

In a confocal microscope, the axial resolution is the smallest separation along the optical axis z between two planes that can still be distinguished. Operationally, it is defined as the full width at half maximum (FWHM) of the system's axial point-spread function (PSF). In reflectance confocal microscopy, illumination and detection share the same objective lens and wavelength, so the axial PSF is set by the product of the illumination and detection intensity distributions, further filtered by the detection pinhole [63].

To obtain the axial response, consider an aberration-free circular pupil of numerical aperture NA focusing into a homogeneous medium of refractive index n . In the Debye–Wolf scalar approximation, a defocus z – i.e. an axial displacement relative to the ideal paraxial focal plane, which is set at $z = 0$ – adds a quadratic phase across the pupil. For normalized pupil radius $\rho \in [0, 1]$, the optical path difference relative to the in-focus reference ray is

$$\text{OPD}(\rho) \approx nz [1 - \cos \theta] \approx nz \frac{\theta^2}{2} \approx z \frac{NA^2}{2n} \rho^2, \quad (2.25)$$

where we used

$$1 - \cos \theta \simeq \theta^2/2 \quad (2.26)$$

$$\theta \simeq \sin \theta \quad (2.27)$$

$$\sin \theta \simeq \rho \sin \theta_{\max} \quad (2.28)$$

$$\sin \theta_{\max} = NA/n. \quad (2.29)$$

Here, θ denotes the polar angle (with respect to the optical axis) of a ray in the object space. In particular, $\theta = 0$ corresponds to the on-axis (central) ray, while $\theta = \theta_{\max}$ corresponds to the marginal ray at the edge of the pupil, with $NA = n \sin \theta_{\max}$. For a uniformly filled circular pupil, a point at normalized pupil radius $\rho \in [0, 1]$ maps to a ray angle such that $\sin \theta \simeq \rho \sin \theta_{\max}$ (and, in the paraxial limit, $\sin \theta \simeq \theta$). The situation is represented in Fig. 2.3.1. If the vacuum wavelength is λ_0 , then the phase is

$$\phi(\rho) = \left(\frac{2\pi}{\lambda_0} \right) \text{OPD}(\rho). \quad (2.30)$$

It is convenient to introduce the dimensionless defocus

$$u \equiv \frac{\pi NA^2}{n \lambda_0} z, \quad (2.31)$$

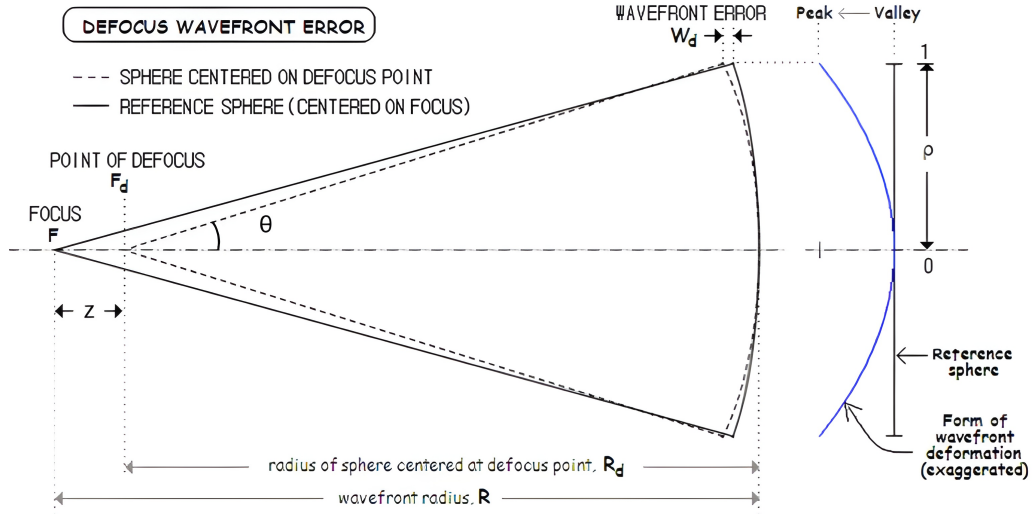


Figure 2.3.1: Schematic representation of axial defocus z relative to the nominal focal plane ($z = 0$), illustrating how an axial displacement introduces a defocus-dependent phase across the objective pupil [64].

so that the phase takes the following form:

$$\phi(\rho) = u \rho^2 \quad (2.32)$$

and the on-axis coherent amplitude is the integral over the circular uniform pupil of the complex phasor:

$$U(z) \propto \iint_{\rho \leq 1} P(\rho) e^{i\phi(\rho)} dA = \int_0^{2\pi} \int_0^1 e^{iu\rho^2} \rho d\rho d\phi. \quad (2.33)$$

The azimuthal integral just gives a constant factor 2π , so up to an overall normalization constant, the problem reduces to a single radial integral:

$$U(z) \propto \int_0^1 e^{iu\rho^2} \rho d\rho = \frac{e^{iu} - 1}{2iu} = e^{iu/2} \frac{\sin(u/2)}{u/2}. \quad (2.34)$$

Hence the widefield on-axis intensity is

$$I_{wf}(z) = |U(z)|^2 = \left[\frac{\sin(u/2)}{u/2} \right]^2, \quad (2.35)$$

represented in Fig. 2.3.2. In a confocal arrangement with an infinitesimal pinhole, the detected intensity from an object point is proportional to the product of the illumination intensity at that point and the collection efficiency of the imaging path. For reflectance,

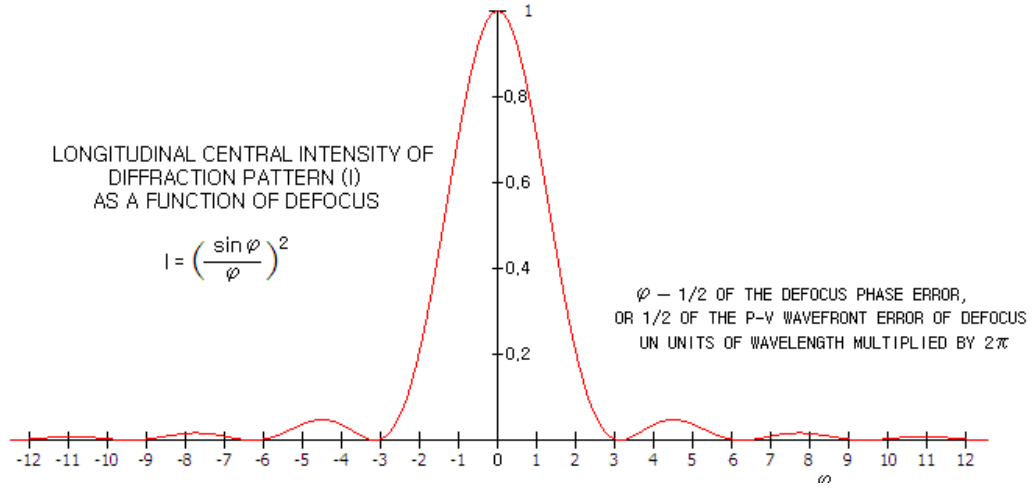


Figure 2.3.2: Illustration of the axial point-spread function (PSF) in confocal microscopy, showing how the intensity varies with defocus [64].

the two paths are identical and at the same wavelength, so the ideal confocal axial response (infinitesimal pinhole) is simply the square of the widefield response:

$$I_{\text{conf},0}(z) \propto I_{wf}(z) I_{wf}(z) = \left[\frac{\sin(u/2)}{u/2} \right]^4. \quad (2.36)$$

By definition, the axial FWHM Δz_0 is the distance between the two axial positions where this response drops to half of its peak. Setting $[\sin(u/2)/(u/2)]^4 = 1/2$ and defining $y \equiv u/2$, one finds that

$$\frac{\sin y}{y} = 2^{-1/4} \approx 0.8409. \quad (2.37)$$

The numerical solution of this equation is $y \simeq 1.0019$. Since $u = (\pi NA^2/(n\lambda_0)) z$ and $u = 2y$ at half-maximum, the half-width is

$$z_{1/2} = \frac{u n \lambda_0}{\pi NA^2} = \frac{2y}{\pi} \frac{n \lambda_0}{NA^2}, \quad (2.38)$$

and the FWHM (twice this) evaluates to

$$\Delta z_0 = \frac{4y}{\pi} \frac{n \lambda_0}{NA^2} \approx 1.28 \frac{n \lambda_0}{NA^2}. \quad (2.39)$$

It is interesting to note that the widefield axial FWHM obtained from $I_{wf}(z) = [\sin(u/2)/(u/2)]^2$ is

$$\Delta z_{wf} \approx 1.77 \frac{n \lambda_0}{NA^2}. \quad (2.40)$$

Real instruments, however, employ a finite detection pinhole of diameter d_p . It is conven-

tional to express this as a number of Airy units

$$s \equiv \frac{d_p}{d_{\text{Airy}}}, \quad d_{\text{Airy}} = \frac{1.22 \lambda_0 M}{NA}, \quad (2.41)$$

where M is the lateral magnification from the specimen to the pinhole plane (equivalently, $d_{\text{Airy}} = 1.22 \lambda_0 f / NA$ in an image plane formed by a lens of focal length f).

A finite-diameter pinhole admits a lateral region of the detection PSF inevitably, letting some out-of-focus light pass and therefore broadening the axial response. To obtain a compact dependence of axial section thickness on pinhole size, the lateral Airy patterns can be modelled both in illumination and detection by rotationally symmetric Gaussians in the same way of Section 2.2. Near focus, each arm's lateral intensity is well described by a Gaussian whose width broadens with defocus and whose on-axis value decreases accordingly (paraxial Debye/Gaussian-beam surrogate). In reflection, the confocal signal at a given axial offset is then proportional to the product of the two co-axial Gaussians, which is again Gaussian, with half the variance and the expected on-axis falloff. The detected signal with a finite circular pinhole is then the encircled energy of this product profile over the pinhole disc (with radius set by the pinhole diameter in Airy units). Normalizing to the in-focus signal yields an axial response that factorizes into a “confocal core” term (from the product of the two defocused Gaussians) and a finite-pinhole acceptance term (the fraction of that product profile admitted by the disc). Expanding the acceptance term for small to moderate pinholes (≈ 0 –1 AU) gives a correction that scales quadratically with the pinhole size. Calibrating the Gaussian-Airy mapping via standard FWHM matching converts that correction into the simple multiplicative law below, which captures how lateral acceptance increases slightly with the defocus s and thus broadens the axial response:

$$\Delta z(s) \approx \Delta z_0 \sqrt{1 + \frac{s^2}{4}}. \quad (2.42)$$

Which is mostly accurate for $s \lesssim 1$ –1.5. It's easy to see that for $s = 1$ (one Airy unit), the multiplicative factor becomes

$$\sqrt{1 + \frac{1}{4}} \approx 1.118 \quad (2.43)$$

And therefore

$$\Delta z(1 \text{ AU}) \approx 1.118 \times 1.28 \frac{n\lambda_0}{NA^2} \approx 1.43 \frac{n\lambda_0}{NA^2}. \quad (2.44)$$

This is the basis for the widely adopted practical formula:

$$\boxed{\Delta z \simeq 1.4 \frac{n \lambda_0}{NA^2}} \quad (2.45)$$

for reflectance confocal operated with a pinhole of about one Airy unit. It is important to mention that, in a real optical system, refractive-index mismatch and imaging depth introduce spherical aberration that elongates the axial PSF. In those cases, the simple scaling $\propto n\lambda_0/NA^2$ underestimates the true optical section thickness. For larger pinholes the simple $\sqrt{1+s^2/4}$ model ceases to be quantitatively accurate and the axial FWHM saturates toward the widefield limit value $\sim 1.77 n\lambda_0/NA^2$. Indeed, the detected signal corresponds to the encircled energy of the squared defocused coherent PSF inside the circular pinhole in the detection plane and its evaluation requires a different analysis. However, a compact closed-form fit that reduces to the infinitesimal-pinhole confocal thickness and its small- s expansion, and saturates to the widefield limit as $s \rightarrow \infty$, is

$$\boxed{\Delta z(s) \approx \sqrt{\Delta z_0^2 + (\Delta z_{wf}^2 - \Delta z_0^2) \frac{s^2}{s^2 + s_t^2}},} \quad (2.46)$$

with $s = d_p/d_{\text{Airy}}$ (Airy units), $\Delta z_0 \simeq 1.28 n\lambda_0/NA^2$, $\Delta z_{wf} \simeq 1.77 n\lambda_0/NA^2$, and a crossover scale $s_t \simeq 1.91$ AU is chosen to match the small-pinhole law $\Delta z(s) \approx \Delta z_0 \sqrt{1+s^2/4}$.

Tightening the pinhole below 1 Airy unit does drive the axial FWHM toward the $\sim 1.28 n\lambda_0/NA^2$ ideal, but at the cost of signal-to-noise ratio [58, 65].

2.4. Sources of Optical Contrast in Skin

As already mentioned, in reflectance confocal microscopy image formation is based on the detection of back-reflected light from a thin optical section within the skin. The fundamental contrast mechanism in RCM is the presence of local refractive index (RI) variations between tissue constituents and their surroundings [66]. When light encounters a boundary between two media with different refractive indices, a portion of the field is reflected according to Fresnel's laws. For an ideal planar interface at normal incidence, the reflectance scales approximately as

$$R \approx \left(\frac{n_1 - n_2}{n_1 + n_2} \right)^2, \quad (2.47)$$

showing that even modest differences in refractive index can generate measurable reflectance. In biological tissue, however, most structures are not planar boundaries but rather heterogeneous assemblies of particles and organelles whose size is comparable to the wavelength; thus, the detected signal is more accurately interpreted as a combination of specular reflection from interfaces and elastic scattering (often in the Mie regime) from subcellular and extracellular microstructures [67]. Importantly, the confocal pinhole suppresses out-of-focus multiply scattered photons, biasing the measurement toward ballistic or quasi-ballistic backscattered light originating near the focal plane [68]. Consequently, RCM contrast is strongly determined by (i) the magnitude of RI mismatches (Δn), (ii) the density and size distribution of scatterers, and (iii) the local organization of tissue (e.g., layered interfaces, fibers, granules) [69]. In clinical RCM, the brightest structures are typically those with a high refractive index and/or high scattering efficiency. Across multiple clinical references and guidelines, the dominant endogenous sources of high reflectance in skin are keratin, melanin, and collagen, along with other sharp boundary layers characterized by strong RI discontinuities. Keratin-rich tissue produces strong backscatter due to its relatively high refractive index and dense microstructure and this keratin-driven brightness is exploited clinically to identify superficial morphology and to recognize characteristic patterns in hyperkeratotic or parakeratotic conditions [70]. An example is shown in Fig. 2.4.1. Melanin is one of the strongest contrast agents in RCM because melanin-containing granules (melanosomes) exhibit a markedly higher refractive index than the surrounding cytoplasm [66]. Reported values commonly place melanin granules around $n \approx 1.7$, compared to cytoplasm around $n \approx 1.35$, producing a large Δn and therefore strong backscatter. This is the physical basis for the characteristic high reflectivity of melanocytes and melanin-rich keratinocytes, and it explains why pigmented lesions often display strong signal and high contrast in the basal layer. Although melanin

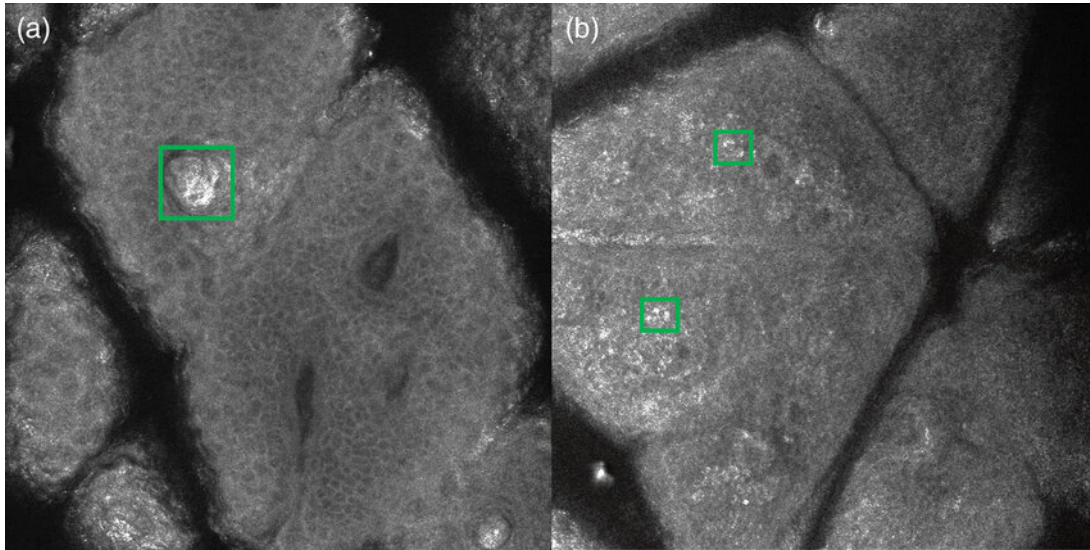


Figure 2.4.1: Example RCM images showing bright spots due to a) keratin in hair shafts and b) clustered bright structures [71].

also absorbs light, at the NIR wavelengths typically used in clinical RCM, the imaging signal is primarily driven by the elastic scattering/reflectance contrast due to refractive-index heterogeneity, with absorption playing a secondary role in shaping intensity [28]. The contrast provided by melanin-rich structures can be appreciated in Fig. 2.4.2. In the superficial dermis, contrast is largely dominated by the extracellular matrix, particularly collagen fibers. Collagen bundles act as efficient scatterers due to their fibrillar morphology and refractive-index differences relative to the surrounding interstitial fluid. Clinically, collagen often appears as a bright fibrous network in the papillary dermis [73]. Equally important for image interpretation are structures that appear dark in RCM. Regions with relatively uniform refractive index and low scattering—such as extracellular fluid spaces—or structures with weaker reflectance relative to their surroundings can produce low signal. A classic example is the cell nucleus, which in many RCM images appears dark relative to the brighter cytoplasm or surrounding keratinized and melanin-rich components [70] as can be seen in Fig. 2.4.3. The set of dominant contrast sources changes with imaging depth, due to both tissue composition and attenuation of ballistic photons. In most clinical deployments, RCM provides useful imaging through the epidermis and into the superficial dermis, with typical penetration depths on the order of 300–400 μm (papillary dermis / upper reticular dermis depending on site and patient). Superficially, keratin-related contrast dominates. Within the living epidermis, cell borders, intracellular granularity, and melanin distribution become prominent. Eventually, in the papillary dermis, collagen fibers and dermal microarchitecture are the main reflectance features. This depth-dependent contrast hierarchy is central to clinical interpretation [66, 70].

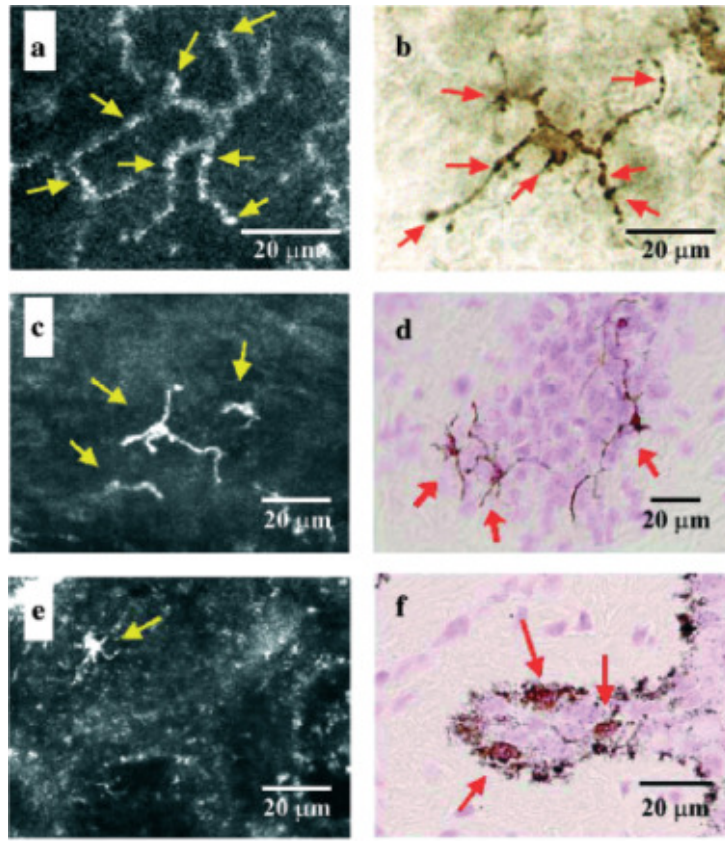


Figure 2.4.2: Example illustrating how melanin, due to its high refractive index and strong backscattering, provides bright contrast in reflectance confocal microscopy images of skin from various animals [72].

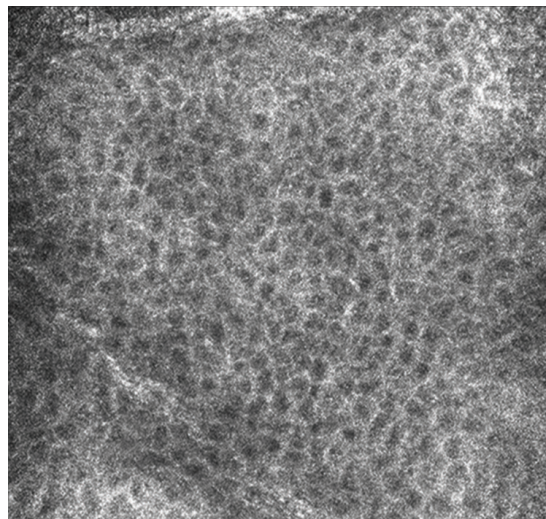


Figure 2.4.3: Example RCM image highlighting the appearance of cell nuclei and their contribution to contrast at the cellular scale [74].

2.5. Scanning Mechanisms in Confocal Systems

In confocal microscopy, the scanning subsystem defines how the diffraction-limited illumination and detection volumes are translated across the specimen to produce the image. Scanner choice directly sets spatial and temporal sampling, optical throughput, and the attainable signal-to-noise ratio, which are critical for in-vivo reflectance imaging of skin. Placing the scanning elements in planes conjugate to the objective pupil preserves object-space telecentricity (constant NA and magnification across the field and minimal pinhole walk-off), and allows straightforward descanning of the return beam to a single-pixel detector. The advent of video-rate reflectance confocal microscopy demonstrated that epidermal and superficial dermal morphology can be visualized non-invasively in real-time and with histologic correlation if an RCM is able to achieve fast enough imaging while preserving image quality, elevating the scanning strategy from an auxiliary detail to a primary determinant of diagnostic performance. A widely adopted baseline employs two galvanometric mirrors arranged in conjugate pupil planes to generate a telecentric raster (“galvo–galvo” point scanning) as shown in Fig. 2.1.2. This configuration offers linear positioning in object space, flexible waveform control (sawtooth or more general trajectories), and straightforward descanning to a single-pixel detector, which maximizes detection efficiency, maintains confocal sectioning, and enables region-of-interest (ROI) zooming via voltage scaling without optical reconfiguration. Limitations arise from inertia: the achievable frame rate is bounded by the slow-axis acceleration and flyback overhead, and excessive slew rates excite mechanical resonances. In practice, the line scan rate and the pixel dwell time must be chosen to balance SNR against motion blur in vivo. Comparative analyses of confocal modalities emphasize these trade-offs between speed, sectioning performance, and flexibility in classical point-scanning systems [75]. A common practical variant replaces the fast galvanometer with a resonant scanner while retaining a conventional galvo for the slow axis [76]. Resonant scanners provide a fixed, high sinusoidal line frequency (typically 8–12 kHz in microscopy), eliminating flyback and delivering excellent line-to-line repeatability with low jitter. The sinusoidal velocity, however, produces non-uniform instantaneous pixel spacing, which is corrected by time-base warping or look-up remapping and ROI zoom is still available by adjusting the slow axis and acquisition window [77]. Replacing the fast axis with a rotating polygon mirror yields a hybrid scheme with very high and highly uniform line rates. Polygon scanners generate nearly constant line velocity and low line-to-line jitter, which reduces geometric distortion and facilitates clock-synchronous acquisition. An f - θ scan lens is typically used to linearize the angle-to-position mapping [79, 80]. Facet wobble and facet-to-facet nonuniformity demand precise optomechanical alignment and phase/timing calibration.



Figure 2.5.1: A resonant scanner, where the mirror oscillates at its resonance frequency, producing a sinusoidal angular sweep and enabling high line rates for fast imaging [78].

The inter-facet retrace gap lowers duty cycle unless bidirectional readout is adopted and the polygon scanner itself adds mechanical complexity [81]. High-speed demonstrations with polygon scanners have reached 512×512 pixels at 200 frames per second, illustrating the attainable performance envelope. Double-reflection polygon designs have also been proposed to maintain constant path length and improve duty cycle in en-face scanning [82]. Parallelized scanning with a spinning Nipkow disk distributes illumination across

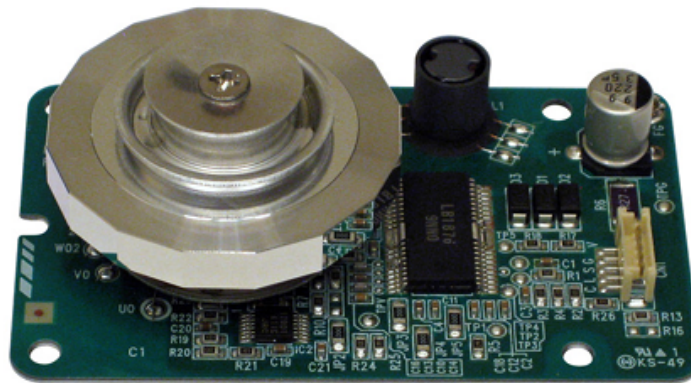


Figure 2.5.2: A polygonal mirror scanner: the rotating multi-facet mirror produces a near-linear angular sweep over each facet, enabling high-speed line scanning for fast imaging [83].

thousands of pinholes and relays the return signal to a camera, enabling high frame rates leveraging turnkey camera detection and high temporal resolution [84]. In thick, scattering tissues such as skin, however, discretized confocality and pinhole cross-talk in-

introduce background that degrades axial sectioning and contrast. Modern developments mitigate these effects: dual-disk designs with microlenses increase throughput and reduce cross-talk, and optical photon reassignment reassigns detected photons to improve lateral resolution in fluorescence [85]. These approaches have achieved extreme frame rates in live-cell imaging, but for label-free reflectance in scattering tissue, point- or line-scanned architectures typically retain superior background rejection and axial sectioning [86]. Line-scanning confocal microscopy (LSCM) offers a pragmatic compromise by illu-

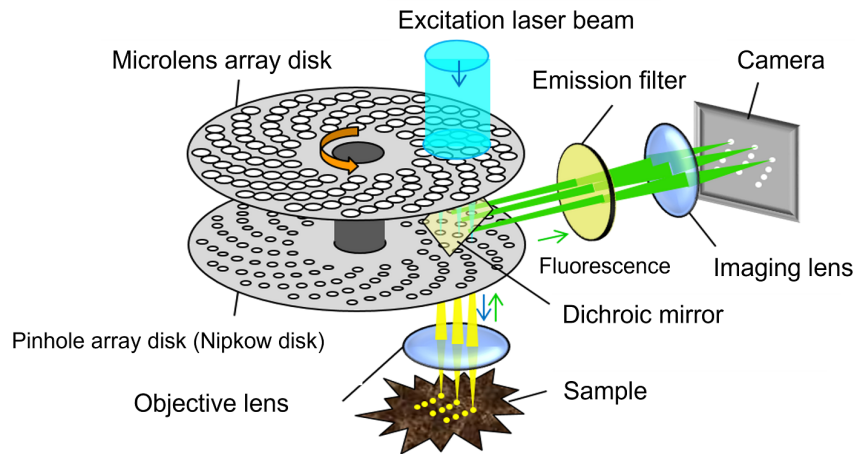


Figure 2.5.3: Illustration of Nipkow disk confocal scanning: an array of pinholes arranged on a rotating disk enables parallel excitation and detection, increasing acquisition speed [87].

minating with a diffraction-limited line rather than a point and detecting with a linear array through a confocal slit or via appropriate pupil engineering. The scanning line can be generated in various ways: from a classical cylinder lens that focuses light only in one lateral direction to more advanced systems, involving grating prisms and supercontinuum lasers (wavelength-based separation). Because only one mechanical axis is scanned, frame rates increase and optomechanics simplify. The predictable penalty is reduced confocality in the direction orthogonal to the line; the effective axial/lateral trade-off is governed by slit width and by how the pupil is partitioned. Practical implementations include acousto-optic deflectors for the scan axis and line cameras or sCMOS sensors for detection [88]. With sCMOS, a rolling shutter synchronized to the scanned line can act as an electronic confocal slit, improving background rejection without a physical slit [89]. Both bench and clinical studies have quantified these trade-offs in human skin, characterizing resolution, sectioning, contrast, and speckle under full-pupil and divided-pupil configurations and demonstrating in-vivo visualization of nuclear and cellular detail in skin and oral mucosa [90]. The line-scan geometry is illustrated in Fig. 2.5.4. Theta line-scanning (θ -LS) augments LSCM by splitting the objective's pupil and orienting illumination and

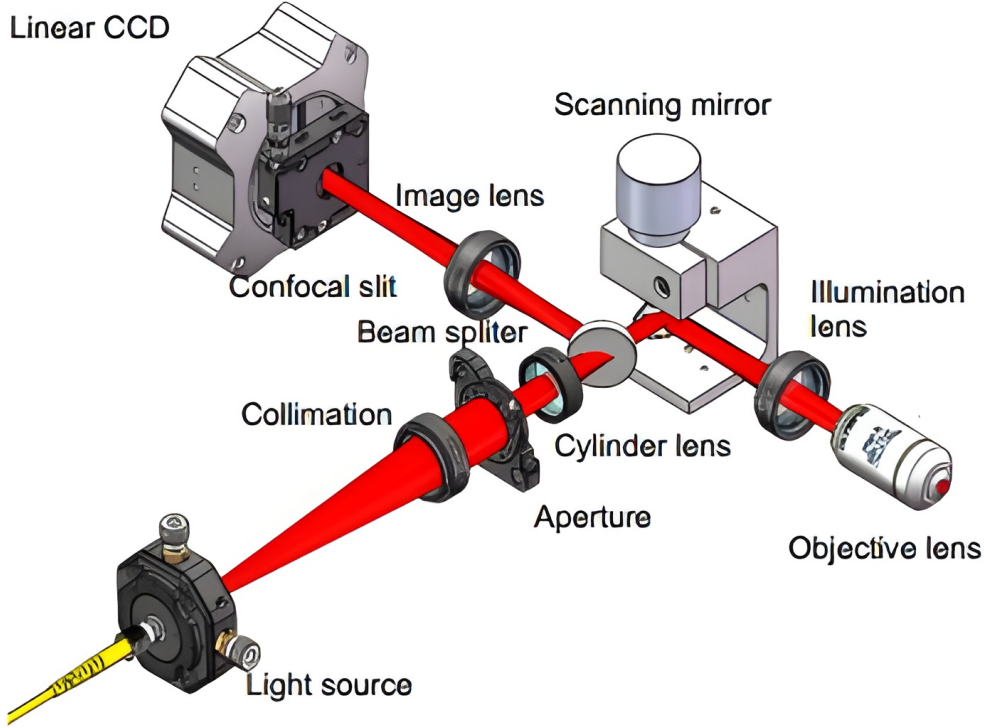


Figure 2.5.4: Schematic representation of line scanning, where the illumination is shaped into a line and swept across the sample to form a two-dimensional image [91].

detection to intersect at a small internal angle θ within the specimen. This separated-pupil geometry suppresses mirror-like back-reflections and improves axial discrimination at the intersection, while retaining the mechanical simplicity of single-axis scanning and linear-array detection. Intuitively, the tilted illumination and detection PSFs intersect in a narrower axial region, recovering much of the sectioning lost in conventional line scanning; the trade-off is reduced optical throughput per arm (each half-pupil carries half the aperture), so sensitivity and noise-equivalent power must be managed within exposure limits. In vivo, θ -LS reflectance systems have shown nuclear-level contrast in epidermis and oral mucosa with relatively few optical components, provided that pupil partitioning and alignment are carefully maintained [30, 89]. The θ line scanning approach is shown in Fig. 2.5.5. Across these implementations, the detection topology and electronics couple directly to scanning. In reflectance confocal microscopy, descanned single-pixel detection (PMT or APD) remains standard because it preserves the pinhole's spatial discrimination and maximizes quantum efficiency at the detector plane. The SNR at a given frame rate is governed by photon statistics and bandwidth: for a fixed optical power and reflectance, SNR scales approximately as

$$\text{SNR} \propto \sqrt{N_{\text{ph}}}, \quad (2.48)$$

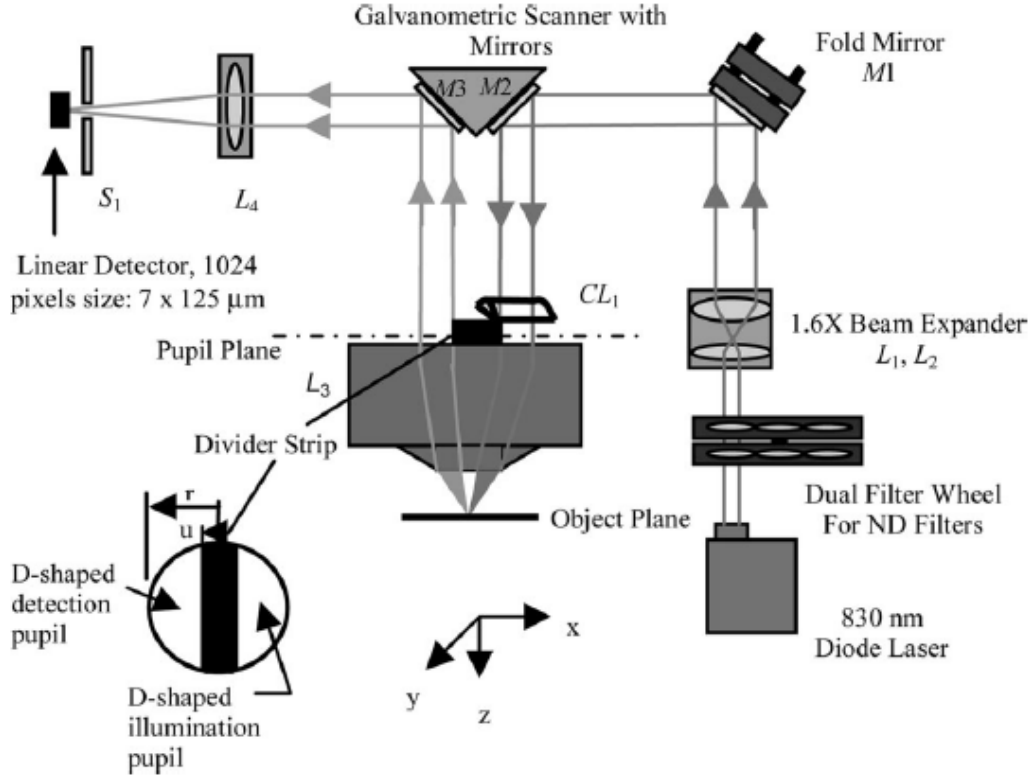


Figure 2.5.5: θ line-scan-based RCM setup scheme [92].

with

$$N_{\text{ph}} \propto \frac{\eta_{\text{det}} P \tau}{h\nu}, \quad (2.49)$$

where η_{det} is end-to-end detection efficiency, P the returned power, and τ the pixel dwell time. Thus, pushing frame rate (shorter τ) reduces SNR unless compensated by higher throughput, higher QE, or optimized pinhole/slit sizing. In scattering skin, maintaining confocal rejection is typically more beneficial than enlarging acceptance beyond 1 AU because out-of-focus background rises rapidly. Overall, galvanometer-based point scanning maximizes confocality and detection efficiency but is speed-limited by inertia; resonant and galvo point scanning removes flyback and reduces jitter at the cost of pixel remapping; hybrid galvo–polygon scanning delivers markedly higher and more linear line rates with added opto-mechanical complexity; spinning-disk approaches excel in live-cell imaging but are disadvantaged in reflectance of scattering tissues by cross-talk and out-of-focus background; line scanning increases throughput and simplifies mechanics with a controlled sacrifice in orthogonal confocality; and θ -line scanning recovers much of the axial sectioning while remaining mechanically terse [93]. For confocal RCM aimed at non-invasive lesion imaging, scanner selection should be driven by the line scan rate required to outrun patient motion and to resolve diagnostically relevant features.

2.6. Pinhole Dimensioning in Confocal Systems

In a confocal microscope the detection pinhole, placed in a plane conjugate to the focal plane, spatially filters the return beam so that only light originating from the vicinity of the focus contributes to the formation of the image. The pinhole therefore shapes the confocal point-spread function (PSF) as the pointwise product of illumination and detection intensity PSFs, controls the degree of out-of-focus rejection (optical sectioning), and has first-order impact on signal throughput, contrast, and noise performance. Classical analyses show that, whereas an infinitesimal aperture maximizes sectioning, it also extinguishes the signal; conversely, enlarging the aperture transitions the instrument toward widefield behaviour with diminished sectioning and contrast. Selecting the pinhole is thus a quantitative compromise tailored to specimen properties and the dominant noise regime [94, 95]. It is convenient to express the pinhole diameter in Airy units (AU), where 1 AU corresponds to the diameter of the in-focus Airy disk at the pinhole plane. For an objective lens of numerical aperture NA and detection wavelength λ_0 imaged onto the pinhole with lateral magnification M , the physical diameter for one Airy unit is

$$d_{\text{AU}} \approx \frac{1.22 \lambda_0 M}{NA}. \quad (2.50)$$

In routine laser-scanning practice, the pinhole is often set near $s = 1$ AU. This preserves most of the confocal sectioning while maintaining usable signal. Closing below 1 AU can improve lateral FWHM (approaching the $\sqrt{2}$ gain as $s \rightarrow 0$) at the cost of rapidly falling signal and SNR, whereas opening above ~ 1 –2 AU progressively degrades axial discrimination and converges toward widefield performance. From Section 2.3, the axial FWHM (optical section thickness) for an infinitesimal pinhole is

$$\Delta z_0 \approx 1.28 \frac{n \lambda_0}{NA^2}, \quad (2.51)$$

and, in the small- to moderate-pinhole regime, grows with the defocus s approximately as

$$\Delta z(s) \approx \Delta z_0 \sqrt{1 + \frac{s^2}{4}} \quad \text{for } s \lesssim 1\text{--}1.5, \quad (2.52)$$

reflecting the fact that a finite aperture admits an increasing portion of the defocused lateral profile [96]. For larger pinholes the axial FWHM saturates toward the widefield value $\Delta z_{\text{wf}} \approx 1.77 n \lambda_0 / NA^2$ [97]. A convenient all- s fit that enforces the correct limits is

$$\Delta z(s) \approx \sqrt{\Delta z_0^2 + (\Delta z_{\text{wf}}^2 - \Delta z_0^2) \frac{s^2}{s^2 + s_t^2}}, \quad \text{where } s_t \simeq 1.91 \text{ AU} \quad (2.53)$$

while the lateral FWHM interpolates from the confocal limit toward the widefield Airy value as s increases (see Section 2.3). The SNR implications of pinhole size have been treated analytically and experimentally. A simple photon-budget model can be used to make the trade-off explicit:

$$\text{SNR} \approx \frac{S(s)}{\sqrt{S(s) + B(s) + N_{\text{det}}^2}}, \quad (2.54)$$

where $S(s)$ is the in-focus signal admitted by a pinhole of size s , $B(s)$ represents the out-of-focus and multiply scattered background admitted by the same pinhole, and N_{det} aggregates detector noise (shot, dark, read, TIA). Under shot-noise-limited conditions with negligible background, $\text{SNR} \propto \sqrt{S(s)}$ would suggest opening the pinhole. In practice, however, $S(s)$ saturates once the central Airy lobe and a few rings are admitted, whereas $B(s)$ typically rises more steeply with s . As a consequence, SNR does not increase monotonically with pinhole size. Both theory and measurement place the fluorescence-imaging optimum near order-unity AU for thin, low-scatter specimens [98, 99]. In in-vivo reflectance confocal microscopy (RCM) of skin, the situation differs for two reasons. First, the specimen's strong elastic scattering introduces a significant multiply scattered background that grows with pinhole size. Second, the coherence of narrowband illumination produces speckle, a granular interference pattern whose statistics can dominate image texture and obscure morphology [100]. The effects of speckle on RCM images quality are shown in Fig. 2.6.1.

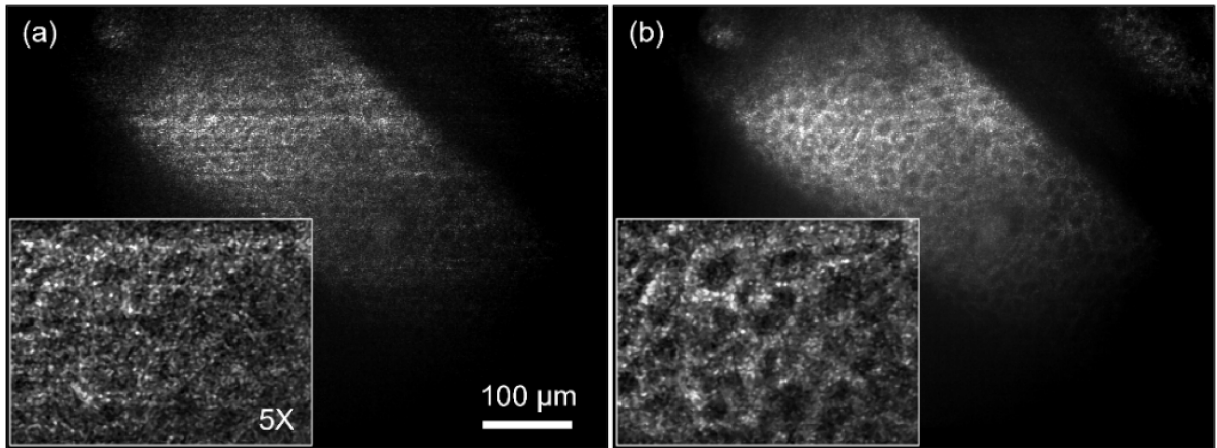


Figure 2.6.1: Example of speckle, a granular interference pattern arising from coherent illumination and multiple scattering, which can reduce contrast and obscure fine structures in the image. RCM images of the same portion of skin on a human finger a) with speckle and b) with speckle reduction [101].

Here the pinhole plays a second role: it is a spatial averaging aperture over the speckle field at the pinhole plane. Admitting N statistically independent speckle grains, speckle contrast decreases approximately as

$$C \sim \frac{1}{\sqrt{N}}. \quad (2.55)$$

Consequently, modestly enlarging the pinhole can markedly improve perceived image quality even if sectioning is modestly reduced. Analyses and experiments specific to RCM report that increasing the detection aperture from the “textbook” ~ 1 AU toward several resolution elements (resels) drives the speckle intensity distribution toward a more normal (less heavy-tailed) form and reduces speckle contrast while maintaining clinically adequate sectioning. Here, one resel corresponds to one object-space lateral resolution element—approximately the lateral FWHM of the confocal PSF at the chosen pinhole setting. A practical operating regime for coherent reflectance imaging is therefore to avoid an aperture equal to a single resel and instead select a diameter on the order of ~ 3 – 5 resels (i.e. several Airy diameters for a circular pupil). This averages multiple independent speckle grains and stabilizes contrast while preserving sufficient axial discrimination to resolve epidermal and superficial dermal features. This rule of thumb is consistent with statistical treatments and with human-tissue RCM measurements that identified apertures of approximately this scale as optimal for speckle suppression without unacceptable loss of sectioning. Complementary strategies—pinhole dithering (lateral shifting), frame summation with small inter-frame decorrelation, or illumination speckle modulation—further reduce speckle and pair well with a moderately enlarged pinhole [31]. The consequences of “opening up” the pinhole size are modality-specific and should be quantified for the instrument at hand. In point-scanning RCM, enlarging the pinhole primarily broadens the axial response and increases multiply scattered background; in line-scanning reflectance systems, the entrance slit plays the analogous role and similar trade-offs apply: wider slits reduce speckle and increase throughput but diminish sectioning. Systematic studies in human skin and oral mucosa show that increasing slit width can reduce speckle more effectively than temporal averaging, while still preserving diagnostically useful nuclear-level contrast, provided that the slit remains within a few AU equivalent [94]. In summary, pinhole selection is best framed as an optimization over three coupled goals: axial sectioning (and with it contrast against out-of-focus background), lateral resolution, and statistical noise properties (SNR and speckle). Foundational theory places the “optimum” near ~ 1 AU for fluorescence imaging of thin, low-scatter specimens under shot-noise-limited conditions; for coherent reflectance imaging of scattering tissues, a somewhat larger aperture—on the order of 3–5 resels—is advantageous to decorrelate

speckle and stabilize contrast, especially when complemented by speckle-reduction tactics such as pinhole dithering or illumination modulation. The corresponding physical diameter follows from NA , λ_0 , and relay magnification M via $d_{\text{AU}} \approx 1.22 \lambda_0 M / NA$. In practice, the chosen setting should be validated empirically by measuring axial response, contrast-to-noise ratio, and speckle contrast on tissue-mimicking phantoms and in vivo, and by confirming that the frame rate and pixel dwell time deliver adequate SNR at the selected aperture.

2.7. Acquisition Speed vs. Image Quality Trade-Off in Reflectance Confocal Microscopy

In raster-scanned confocal microscopy, the desired frame rate fixes the pixel dwell time τ_{px} (the integration time per pixel), which in turn sets the mean number of detected photo-events per pixel N_{phot} and thus the achievable signal-to-noise ratio (SNR). Under shot-noise-limited detection,

$$\text{SNR} \propto \sqrt{N_{phot}} \propto \sqrt{\tau_{px}}, \quad (2.56)$$

so increasing the frame rate (shorter τ_{px}) degrades SNR unless compensated by illumination, detection efficiency, or averaging [102]. In vivo reflectance confocal microscopy (RCM) adds a competing requirement: sufficiently high video-rate acquisition (≈ 10 fps or higher) helps “freeze” tissue/patient motion and enables mosaicking with minimal motion artefacts. The operating point is therefore a balance between motion robustness and photon-limited noise. For a raster of $N_x \times N_y$ pixels at frame rate f_F with scan duty factor $\delta \in (0, 1]$ (fraction of time actually scanning, i.e., excluding flyback), the per-pixel dwell time is

$$\tau_{px} \approx \frac{\delta}{f_F N_x N_y}. \quad (2.57)$$

Example: 1000×1000 , $f_F = 10$ fps, $\delta = 0.9 \Rightarrow \tau_{px} \approx 9.0 \times 10^{-8}$ s ≈ 90 ns. Since

$$N_{phot} \propto \frac{\eta_{det} P \tau_{px}}{h\nu}, \quad (2.58)$$

(with η_{det} the end-to-end detection efficiency and P the returned power at the detector plane), SNR scales as $\sqrt{\tau_{px}}$ at fixed P and η_{det} [103]. Scanner choice affects not only speed but also geometric fidelity of the raster. Resonant fast-axis scanners produce a sinusoidal line motion at fixed frequency; pixel positions are linearized by time-base warping or LUT remapping (there is no flyback penalty) [76]. Polygon scanners deliver nearly constant line velocity with very low intra-line jitter (often with an f - θ lens to linearize angle-to-position mapping) [80]. Galvo-galvo pairs offer flexible waveforms and ROI zoom at lower accelerations but incur flyback. As the line scan rate increases, the detector/transimpedance-amplifier (TIA) and anti-alias filters become limiting: for a first-order (single-pole) front-end, a useful rule-of-thumb is

$$f_{-3\text{dB}} \gtrsim \frac{0.35}{\tau_{px}} \quad (2.59)$$

to avoid signal attenuation and intersymbol interference at the pixel timescale. Higher readout speeds tighten timing tolerances and raise electronic-noise contributions. Comparative reviews emphasize that faster scanning improves temporal resolution but, all else equal, reduces per-pixel exposure and thus SNR. Compensation for the SNR penalty at high speed follows three main routes, each with consequences for image quality and safety: (i) Increase illumination: it raises N_{phot} but it is constrained in vivo by ocular/skin exposure standards and, in fluorescence, by photobleaching/phototoxicity. In label-free reflectance at 830 nm, powers of $\sim 1\text{--}5$ mW at tissue are typical and considered safe for clinical RCM [66]. Within these bounds, SNR is primarily managed via detector sensitivity, pinhole/slit setting, and modest frame averaging. (ii) Increase throughput/efficiency: improve optical coupling and quantum efficiency, and choose the pinhole size near the regime that balances sectioning against SNR and contrast. Slightly enlarging the pinhole (from ~ 1 AU toward a few resels) increases detected photons and reduces speckle variance at the expense of some axial sectioning—often a favorable trade at video-rate in scattering skin (Section 2.6). (iii) Average in time/space: multi-frame averaging restores the $\sqrt{N}$ dependence for the SNR if the inter-frame motion is corrected (line- or frame-wise registration). Spatial binning is also applicable to camera/line-scan detection. Faster scanning can mitigate photobleaching in high-irradiance fluorescence by reducing residence time, but it does not change the fundamental $\sqrt{\tau_{px}}$ SNR scaling in confocal detection. Backscattered intensity statistics and coherence strongly shape the speed–quality compromise in reflectance imaging [103]. Speckle noise from coherent backscatter dominates texture in RCM and does not average out within a single short-exposure frame. Strategies that moderate speckle variance—e.g., modestly enlarging the detection pinhole beyond a single resolution element, pinhole dithering (lateral shifts between frames), or averaging a small number of registered frames with gentle decorrelation—measurably improve perceived image quality at a given frame rate, as shown by analyses of speckle contrast versus pinhole size and by pinhole-shifting methods [94] discussed in Section 2.6. In practice, operating near video-rate while using moderate pinhole enlargement has proven effective in vivo for preserving cellular-level contrast with motion robustness. A practical way to set a design target is to budget blur and contrast. To ensure that the dynamic nature of a specimen does not degrade the spatial resolution in the image, a relationship between the specimen velocity and the acquisition speed must be established. Let the specimen drift at a constant velocity v within the object space. During the acquisition period of a single frame, T_F , the specimen undergoes a lateral displacement Δx , defined as:

$$\Delta x = vT_F = \frac{v}{f_F} \quad (2.60)$$

where f_F represents the frame rate of the system. Now let the permissible intra-frame motion blur be a fraction α of the pixel size p (typically $\alpha \approx 0.25$). To maintain image fidelity, the displacement must satisfy the following condition:

$$\Delta x \leq \alpha p \quad (2.61)$$

By substituting the expression for Δx and rearranging for the frame rate, we derive the minimum required temporal frequency:

$$f_F \gtrsim \frac{v}{\alpha p} \quad (2.62)$$

This inequality suggests that for a given pixel size, any increase in specimen velocity requires a proportional increase in frame rate to prevent resolution loss due to motion artifacts. However, it should be noted that increasing f_F to meet this kinematic bound often forces τ_{px} into the sub-microsecond regime at standard fields of view, hence the need to recover SNR by (i) maximizing η_{det} and detector QE, (ii) operating the pinhole in the speckle-averaging sweet spot for reflectance, and (iii) using registration-aware frame summation [103]. On the electronics side, one should set analog bandwidth and digitizer ENOB considering the required τ_{px} to avoid SNR losses from front-end roll-off and quantization noise [102]. In summary, pushing toward ~ 10 fps video-rate improves motion tolerance and workflow in in-vivo RCM but shortens τ_{px} and erodes photon SNR unless compensated. Resonant or polygon fast-axis scanners, high-QE detectors with bandwidth matched to τ_{px} , and pinhole sizing informed by reflectance-specific speckle statistics (rather than fluorescence-centric 1 AU rules) are effective levers. Within in-vivo safety limits at 830 nm, most clinical systems maintain diagnostic cellular contrast at video-rate by combining moderate pinhole enlargement or line-slit tuning with light multi-frame averaging and motion-robust scanning/registration.

2.8. Penetration Depth vs. Optical Resolution Trade-Off in Reflectance Confocal Microscopy

In reflectance confocal microscopy, penetration depth is limited by (i) scattering and absorption, which attenuate the quasi-ballistic signal, and (ii) specimen-induced aberrations, which broaden the point-spread function (PSF) and reduce contrast [28, 29]. In skin, multiply scattered light rises rapidly below the basal layer, while refractive-index-mismatch-driven spherical aberration accumulates with depth. Together they reduce both signal and effective axial/lateral resolution [29]. In vivo, routine RCM typically reaches depths on the order of 200–300 μm , with progressively declining resolution and contrast [21, 28]. Two design variables link depth and resolution most directly: wavelength and numerical aperture (NA). Tissue scattering decreases with wavelength approximately as a negative power law, $\mu'_s(\lambda_0) \propto \lambda_0^{-b}$ ($b \sim 0.7\text{--}1.5$) [46], shown in Fig. 2.8.1.

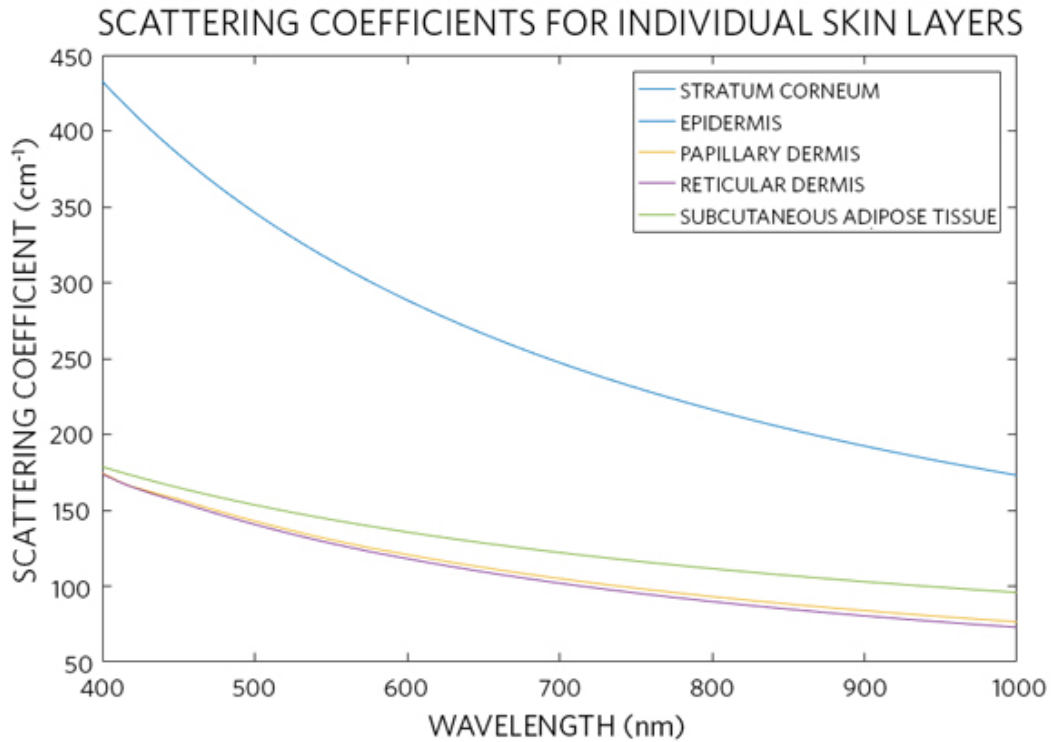


Figure 2.8.1: Illustration of the scattering coefficient μ_s as a function of wavelength, showing the decrease in scattering at longer wavelengths as a negative power law [104].

In epi-confocal geometry, the quasi-ballistic component experiences an effective two-pass attenuation approximately $\propto \exp[-2(\mu_s + \mu_a)z]$ [105]. Thus, increasing λ_0 reduces scattering and can extend depth, but it also enlarges the diffraction-limited focus. Near the surface, the lateral and axial confocal FWHM scale approximately as $\Delta x \approx 0.4 \lambda / \text{NA}$ and $\Delta z \approx 1.28 n \lambda / \text{NA}^2$ [106, 107], see Section 2.2 and 2.3. Clinical RCM systems therefore

often operate in the near-IR around 830 nm as a compromise between reduced scattering, adequate melanin contrast, and acceptable diffraction blur [21]. Near the surface, increasing NA improves lateral resolution and axial sectioning; with depth, however, high- NA focusing becomes increasingly sensitive to refractive-index mismatch, which introduces depth-dependent spherical aberration and PSF broadening [108, 109]. Analyses of index-mismatch aberrations also show how spherical aberration can strongly penalize penetration in practice [110]. Aberration penalties can be reduced by moderating the effective aperture and by using index-matching strategies such as water-immersion objectives and coupling gels. In practice, a moderate NA (e.g., water immersion, $n \approx 1.33$) combined with near-IR illumination often yields better net performance at depth than pushing to the highest nominal NA [21]. Adaptive optics (AO) can further recover contrast and resolution by correcting low-order and specimen-induced aberrations [111]; however, the ultimate depth limit is still set by multiple scattering in tissue [112]. Beyond scattering, the choice of wavelength is further constrained by the absorption spectra of endogenous chromophores. In the visible spectrum, imaging depth is severely limited by the high absorption of hemoglobin and melanin. However, the 'optical window' in tissue (NIR-I, approx. 700–900 nm) represents a spectral region where the absorption coefficients μ_a of water and blood are simultaneously minimized, and so the skin absorption coefficient. While shifting further into the short-wave infrared (SWIR or NIR-II, 1000–1700 nm) can further reduce scattering, the rising absorption of water—particularly the peak near 1450 nm—imposes a trade-off, as it can lead to photothermal heating and signal attenuation. Consequently, 830 nm remains a 'sweet spot' for RCM because it maintains high sensitivity to melanin—which retains a significant backscattering cross-section—while benefiting from the low absorption of the surrounding dermis. The Fig. 2.8.2 shows more clearly the skin optical window in the NIR range 700–900 nm.

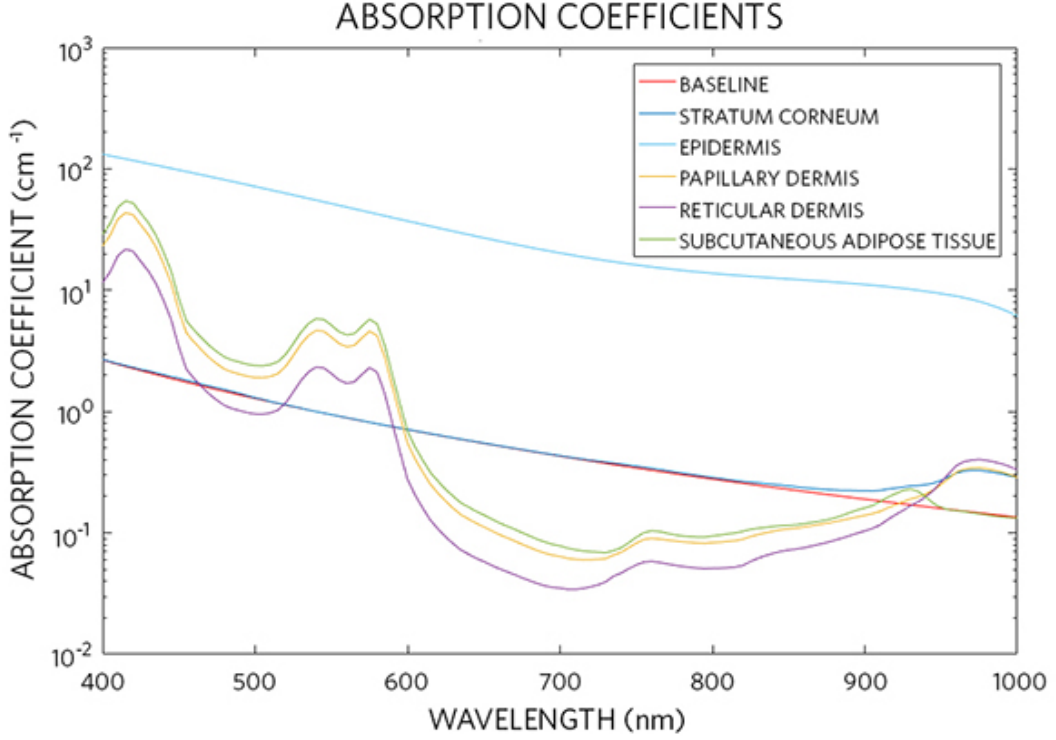


Figure 2.8.2: Illustration of the absorption coefficient μ_a as a function of wavelength in skin, showing the biological tissue NIR optical window [104].

The increased absorption coefficient outside of the optical window directly impacts the signal intensity and consequently the achievable signal-to-noise ratio (SNR) of the system. In the shot-noise-limited regime characteristic of RCM, the SNR scales with the square root of the number of detected photons, $SNR \propto \sqrt{N}$. Since, as previously mentioned, the ballistic signal $N(z)$ decays exponentially with depth due to the total attenuation coefficient μ_t due to both absorption and scattering, the SNR likewise undergoes an exponential degradation too:

$$SNR(z) \propto \sqrt{N_0 \exp(-2\mu_t z)} = \sqrt{N_0} \exp(-\mu_t z) \quad (2.63)$$

where N_0 is the photon count at the surface. Consequently, even a moderate increase in the absorption coefficient μ_a —due to water absorption at longer NIR wavelengths or hemoglobin in the visible range—drastically reduces the available signal, causing the shot noise to dominate and effectively setting the fundamental penetration limit of the instrument. These depth–resolution trade-offs motivate common RCM design choices, including near-IR wavelengths, water-immersion objectives, and careful optimization of the detection aperture and system signal-to-background ratio [21].

3 | Opto-Mechanical Design of the Prototype

3.1. Performance Metrics and Requirements

The performance metrics that guided the system design were defined to address dermatological clinical use-cases, where cellular morphology (e.g., nuclei, keratinocytes, and melanocytic structures) must be visualized with high contrast over clinically relevant fields of view, at video-rate, and down to the papillary and upper reticular dermis. The prototype developed in this thesis was therefore designed to meet the following requirements:

- **Lateral resolution:** A lateral resolution on the order of $1\ \mu\text{m}$ is required to resolve individual cells and nuclear details relevant for diagnosis. For diffraction-limited confocal imaging, the lateral full width at half maximum (FWHM) scales approximately as $\approx 0.4\lambda_0/\text{NA}$ (Sec. 2.2). With an appropriate numerical aperture and near-infrared illumination wavelength, $\approx 0.5\text{--}1\ \mu\text{m}$ is reached in state-of-the-art clinical systems.
- **Optical sectioning (axial resolution):** To isolate en-face planes and suppress out-of-focus scattering, the axial FWHM (optical section thickness) must be of the order of few μm . For small pinholes, the confocal axial FWHM scales as $\approx 1.4n\lambda_0/\text{NA}^2$ (Sec. 2.3), and in practice the achievable sectioning depends on the pinhole diameter (Sec. 2.6). A target of a few μm is set, maintained to the maximum depth permitted by the signal-to-noise ratio.
- **Field of view (FOV):** A single-frame FOV of $0.5\ \text{mm} \times 0.5\ \text{mm}$ on skin was set as a target to match clinical standards of commercial reflectance confocal microscopy instruments (e.g., VivaScope 1500 reports a $0.5\ \text{mm} \times 0.5\ \text{mm}$ frame).
- **Penetration depth:** In vivo clinical RCM operated at $\sim 830\ \text{nm}$ typically reaches $\sim 200\text{--}300\ \mu\text{m}$, with contrast and resolution progressively degrading beyond $\sim 100\text{--}150\ \mu\text{m}$ due to scattering and sample-induced aberrations. Since scattering decreases at longer wavelengths (Sec 2.8), a longer illumination wavelength can improve the maximum achievable imaging depth. For this reason, the present design targets a penetration depth of $\sim 300\text{--}450\ \mu\text{m}$ by using a laser at $\lambda_0 = 980\ \text{nm}$, accepting the trade-off of increased diffraction blur.
- **Imaging speed:** To reduce motion artefacts arising from patient and tissue movement, and to enable video-mosaicking for diagnostic support, frame rates of the order of 10 fps are desirable and have been demonstrated in clinical RCM devices and in video-rate prototypes.
- **z -stack capability:** To acquire depth-resolved information, the prototype inte-

grates a motorized linear translation stage mechanically coupled to the objective lens. By translating the objective along the optical axis (i.e., approximately normal to the skin surface), the focal plane can be shifted to different depths within the epidermis and superficial dermis, enabling acquisition of a z -stack of en-face confocal images. Such volumetric imaging can provide clinically meaningful information, as depth-dependent morphological changes within a lesion may be critical for diagnosis. The clinically useful axial scanning range, which this prototype aims to achieve, is ≈ 0.5 mm.

All together, these specifications define the key performance metrics that have driven the optical and mechanical design of this thesis. In Sec. 3.2, their impact on the selection of λ_0 , NA, pinhole diameter, detector bandwidth and other components is discussed.

3.2. Design and Dimensioning of the Prototype

The performance metrics defined in Section 3.1 drive two first-order design choices: (i) the illumination wavelength λ_0 and (ii) the objective lens parameters, namely numerical aperture (NA), immersion medium, and working distance (WD). As already seen in Section 2.8, in biological tissues the reduced scattering coefficient $\mu'_s(\lambda)$ decreases with wavelength approximately following a negative power law, $\mu'_s(\lambda) \propto \lambda^{-b}$, where the exponent b typically lies in the range ~ 0.7 – 1.5 depending on the tissue layer. Consequently, shifting the operating wavelength from the conventional clinical RCM value of ~ 830 nm toward ~ 1 μm can increase the maximum usable imaging depth, at the expense of increased diffraction blur. In an epi-confocal configuration, the quasi-ballistic return from a depth z undergoes an approximate two-pass attenuation,

$$I(z) \propto \exp[-2(\mu_a + \mu_s)z], \quad (3.1)$$

where μ_a and μ_s are the absorption and scattering coefficients, respectively. Based on this trade-off, the illumination wavelength selected for this prototype is $\lambda_0 = 980$ nm, chosen to reduce scattering and to reach deeper into the papillary and upper reticular dermis. A relevant counter-effect at this wavelength is the presence of a water absorption overtone band around 970–980 nm; thus, operation at 980 nm entails a modest increase in attenuation compared with 830 nm. However, for the optical path lengths and mW-level powers typical of in vivo RCM, this additional absorption remains acceptable. Moreover, 980 nm remains compatible with high-quantum-efficiency silicon detectors. Overall, the choice of 980 nm is a deliberate compromise: it relaxes scattering-limited depth while maintaining clinically reasonable optical throughput. To mitigate refractive-index-mismatch-induced spherical aberration and to reduce Fresnel reflections at the skin interface, a water-dipping objective was selected. Specifically, the prototype employs a Nikon *CFI Apo NIR 40 $\times$ W* objective (NA = 0.80, WD = 3.5 mm, water dipping) (see Appendix A.1). The high NA provides a large collection angle for backscattered photons, while the 3.5 mm working distance accommodates the integration of a contact window. The objective is specified for operation without a coverslip (cover-glass correction = 0), has a back aperture diameter of 8 mm, and is designed for high transmission in the near-infrared range. For completeness, the complete datasheet of the 980 nm laser and of the objective lens, as well as the datasheets of all the other optical and electronic components are reported in Appendix A. Using the results derived in Section 2.2 and 2.3, the near-surface confocal resolutions can be estimated assuming $n \simeq 1.33$ (water) and NA = 0.80.

- **Lateral resolution:**

$$\Delta x_{\text{conf}} \approx 0.40 \frac{\lambda_0}{\text{NA}} \approx 0.40 \frac{980 \text{ nm}}{0.80} \approx 0.49 \text{ } \mu\text{m}. \quad (3.2)$$

This comfortably satisfies the $\sim 1 \text{ } \mu\text{m}$ cellular-resolution requirement, while leaving margin for degradation due to tissue scattering and specimen-induced aberrations.

- **Axial resolution / optical sectioning:**

$$\Delta z_{\text{conf}} \approx 1.4 n \frac{\lambda_0}{\text{NA}^2} \approx 1.4 \times 1.33 \frac{980 \text{ nm}}{0.80^2} \approx 2.9 \text{ } \mu\text{m}. \quad (3.3)$$

With a moderately large pinhole (on the order of a few Airy units), the axial FWHM broadens slightly but remains within the required few-micrometre regime.

These estimates apply to surface-proximal imaging under diffraction-limited conditions. With increasing depth, both effective lateral resolution and optical sectioning broaden due to multiple scattering and to mismatch-induced aberrations. In this context, the water-dipping configuration helps suppress low-order aberrations at the tissue interface, thereby preserving confocal sectioning deeper into tissue. At 980 nm, melanin absorption is weaker than at 830 nm, which can reduce melanin-driven intrinsic contrast while also decreasing superficial attenuation. In RCM, contrast additionally arises from refractive-index mismatches (e.g., keratin and collagen) and from the confocal rejection of out-of-focus scattered light; thus, the combination of $\text{NA} = 0.80$ and water immersion is advantageous for maximizing the quasi-ballistic signal at shallow-to-intermediate depths. Relative to conventional 830 nm clinical systems, the combination of $\lambda_0 = 980 \text{ nm}$ and an $\text{NA} = 0.80$ water-dipping objective is designed to (i) preserve sub-micron lateral resolution near the surface, (ii) provide axial sectioning of a few micrometres, and (iii) bias performance toward greater usable depth by reducing scattering, while accepting a modest increase in water absorption near 970–980 nm. These choices establish the basis for the scanning architecture, relay optics, and detection strategy developed in Sec. 3.3.

3.3. Optical Elements Selection and Prototype Overview

This section describes the illumination source, beam delivery and scanning architecture, polarization-based separation of illumination and detection paths, relay optics, and the confocal detection chain implemented in the RCM prototype. The design choices are explicitly tied to the performance targets defined in Section 3.1 and to the first-order selections of illumination wavelength and objective lens discussed in Section 3.2. The selected objective (Nikon *CFI Apo NIR 40× W*) has an effective focal length $f_{\text{obj}} = 5$ mm. To span a specimen field of view (FOV) of $0.5 \text{ mm} \times 0.5 \text{ mm}$, the required half-field is $x_{1/2} = 0.25$ mm. Therefore, as shown in Fig. 3.3.1, the corresponding object-space half-angle at the objective is

$$\beta = \arctan\left(\frac{x_{1/2}}{f_{\text{obj}}}\right) \approx \arctan\left(\frac{0.25}{5}\right) \approx 2.9^\circ. \quad (3.4)$$

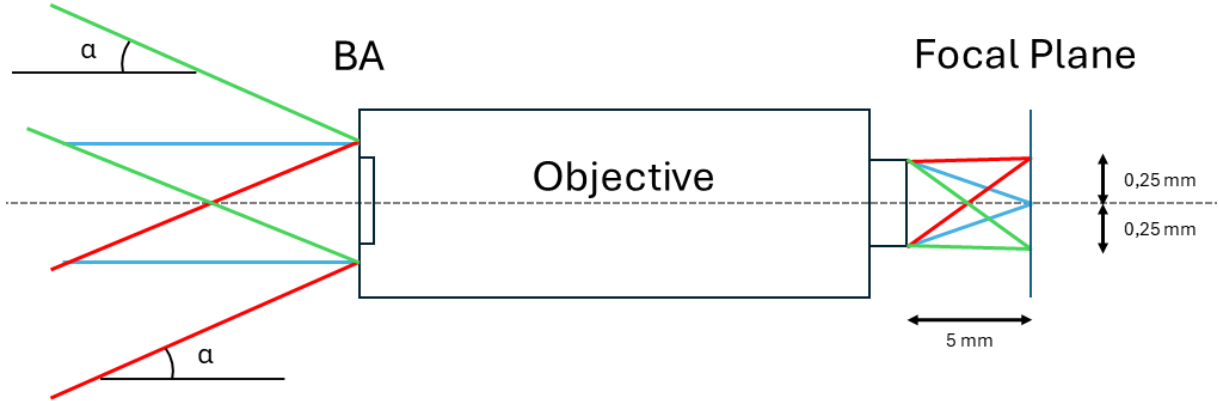


Figure 3.3.1: Optical scheme of the sample scanning.

With numerical aperture $\text{NA} = 0.80$, the objective back-aperture diameter is

$$d_{\text{BA}} = 2 \text{NA} f_{\text{obj}} \approx 2 \times 0.80 \times 5 \text{ mm} \approx 8 \text{ mm}. \quad (3.5)$$

To fully exploit the objective NA while reducing sensitivity to beam walk-off, the illumination beam is designed to overfill the back aperture by approximately 25–30%. Adopting 25% overfill yields a target beam diameter at the objective back aperture of

$$d_{\text{beam,BA}} \approx 1.25 d_{\text{BA}} \approx 10 \text{ mm}. \quad (3.6)$$

The illumination source is a free-space diode laser module (CNI Laser, MDL-III-980L) specified for $\lambda_0 = 980 \pm 10$ nm continuous-wave operation, near-TEM₀₀ spatial mode, $1/e^2$ beam diameter ≈ 3.5 mm at the output aperture, vertical linear polarization, maximum output power of 200 mW, and full-angle divergence < 1 mrad (see Appendix A.2). These parameters provide sufficient optical power for in vivo reflectance imaging and enable expansion to the pupil-filling diameter required at the objective. If the output beam were circular with diameter 3.5 mm, the required beam-expansion magnification to reach $d_{\text{beam,BA}} = 10$ mm would be

$$M = \frac{10 \text{ mm}}{3.5 \text{ mm}} \approx 2.86. \quad (3.7)$$

However, a beam-profile measurement performed at the laser output using the camera DCC1545M from Thorlabs (see datasheet in Appendix A.16 revealed a markedly elliptical mode, as shown in Figure 5.1.2. Using the camera sensor geometry (1280×1024 pixels with $5.2 \mu\text{m}$ pixel pitch), the minor-axis diameter was estimated to be ~ 1.5 mm, while the major axis was found to be ~ 3.5 mm. In the absence of dedicated beam circularization (e.g., cylindrical optics), the minor axis becomes the limiting dimension for pupil filling, and the required overall magnification increases to

$$M_{\text{tot}} = \frac{10 \text{ mm}}{1.5 \text{ mm}} \approx 6.67. \quad (3.8)$$

To meet the video-rate imaging goal, a fast horizontal scanner is required. The fast scan is therefore implemented with a rotating polygon mirror (Precision Laser Scanning, Gecko-45-HSS, Appendix A.3) with $N = 16$ facets and a specified clear aperture of $7 \text{ mm} \times 4.5 \text{ mm}$ per facet. At the maximum rotation speed of 45,000 rpm, the achievable line-scan rate is

$$f_{\text{line}} = \frac{45,000}{60} N \approx \frac{45,000}{60} \times 16 \approx 12 \text{ kHz}. \quad (3.9)$$

Assuming a typical clinical image dimension of 1000×1000 pixels (i.e., ~ 1000 lines per frame), the corresponding frame rate is

$$f_{\text{frame}} \approx \frac{f_{\text{line}}}{1000} \approx 12 \text{ fps}, \quad (3.10)$$

which is of the order of this thesis target of ~ 10 fps for video-mosaicking implementation. The slow vertical scan is implemented with a galvanometric mirror (Thorlabs GVS001, Appendix A.4). While the mirror dimensions are specified as $10 \text{ mm} \times 8 \text{ mm}$, the manufacturer recommends operating with beam diameters $\lesssim 5$ mm on the mirror to preserve margin against clipping across the usable scan range. The GVS001 can provide a maximum mechanical scan angle of $\pm 12.5^\circ$, corresponding to an optical deflection of

$\pm 25^\circ$. The illumination path includes two 4- f relay systems: (i) a relay between the fast polygon scanner and the slow galvo scanner, and (ii) a relay between the slow scanner and the objective back aperture. Because the laser major axis at the source (~ 3.5 mm) is already comparable to the polygon facet height (4.5 mm), the polygon is placed first in the chain to reduce the risk of facet clipping upon beam divergence. Consistently with the recommended $\lesssim 5$ mm beam diameter on the galvo, the first relay (polygon $\rightarrow$ galvo) is designed for unitary magnification, while the beam-expansion factor is fully implemented in the second relay (galvo $\rightarrow$ objective). Both relays employ Thorlabs achromatic doublets (Appendix A.5) rather than singlets to improve wavefront quality and reduce off-axis aberrations during the scanning process. The first relay uses two lenses with focal length $f = 30$ mm separated by $2f = 60$ mm, yielding $1\times$ magnification. The second relay uses $f_1 = 30$ mm and $f_2 = 150$ mm separated by $f_1 + f_2 = 180$ mm, yielding

$$M = \frac{f_2}{f_1} = \frac{150}{30} = 5. \quad (3.11)$$

For the desired 25% overfill of the objective back aperture, a magnification factor of $6.67\times$ is needed. However, the requirements on the total vertical angular scan that are explained below impose a diameter of 2 inches for the second lens of the magnifying 4- f system to relay the entire beam correctly even at the largest scan angles. And being the only 2-inches-diameter lens available the one with $f_2 = 150$ mm, this component was the chosen one. All the other relay lenses have a 1 inch diameter to accommodate the beam over the useful scan range before its magnification. Together with the $5\times$ beam diameter magnification, the second 4- f relay system introduces also an angular demagnification

$$m_\theta = \frac{f_1}{f_2} = \frac{30}{150} = 0.2. \quad (3.12)$$

Therefore, to achieve the required half-angle $\alpha = \pm 2.9^\circ$ at the objective entrance pupil, the scanners must provide a larger input half-angle

$$\alpha = \frac{\beta}{m_\theta} \approx \frac{2.9^\circ}{0.2} \approx 14.5^\circ. \quad (3.13)$$

For the polygon, the facet-to-facet angle is

$$\Delta\phi = \frac{360^\circ}{16} = 22.5^\circ, \quad (3.14)$$

and the corresponding peak-to-peak optical deflection (twice the change in facet normal) is approximately

$$\Delta\theta_{\text{opt,max}} \simeq 2 \Delta\phi \simeq 45^\circ, \quad (3.15)$$

although in practice only a subset of this range is used to avoid edge-related vignetting and clipping. Separation between illumination and detection is implemented via a polarization beam splitter (PBS) and a quarter-wave plate (QWP), as introduced in Section 2.1. The laser output light is specified as vertically polarized but, to further refine the polarization state purity, a linear polarizer is inserted just outside the laser head. A 0.5 inch Thorlabs PBS (Appendix A.6) is oriented to transmit the forward beam (assigned to p -polarization, $\sim 90\%$ transmission). A 1 inch-diameter zero-order QWP from Thorlabs (Appendix A.7) is placed immediately before the objective back aperture, so that circular polarization is confined primarily to the objective/sample region. After a double passage through the QWP, the backscattered light returns with orthogonal linear polarization (which means horizontal polarization) and is reflected by the PBS toward the detection arm (assigned to s -polarization, $\sim 95\%$ reflection), maximizing detection efficiency in a technique with inherently low collection efficiency (typically $\sim 10^{-6}$, depth-dependent). The returned light is collected by the objective, propagates back through the relays, and is de-scanned by the two scanners, so that it returns to the PBS as a stationary collimated beam directed toward the confocal pinhole and detector. To let the illumination beam be transmitted by the PBS (lower efficiency) and the backscattered beam be reflected (higher efficiency), the PBS needs to be oriented accordingly. In this way, the backscattered light is reflected downwards by the PBS. For this reason, a 45° mirror is placed just below the PBS to re-direct the beam towards the detection arm parallel to the optical table. Another lens with $f = 35$ mm focuses the beam onto the confocal pinhole. For a lens of focal length f , the Airy-disk diameter at the focal plane can be expressed as:

$$d_{\text{Airy}} = 2.44 \frac{\lambda f}{d_{\text{eff}}}, \quad (3.16)$$

where $d_{\text{eff}} = \min(d, D)$ is the effective clear beam diameter at the lens, limited by the beam diameter d or the lens clear aperture D . In Section 2.2, this diameter was expressed in terms of the numerical aperture NA under the paraxial approximation for small angles:

$$NA \approx \frac{d_{\text{eff}}}{2f}. \quad (3.17)$$

By substituting this into the initial equation, the diameter of the Airy disk can be rewritten in its standard microscopy form:

$$d_{\text{Airy}} = 1.22 \frac{\lambda}{NA}. \quad (3.18)$$

Note that the coefficient change from 2.44 to 1.22 arises because NA is defined based on the aperture radius ($d_{\text{eff}}/2$), whereas the original formula uses the full diameter d_{eff} . In the present architecture, the return beam diameter is set by the demagnification between the objective back aperture and the galvo:

$$d_{\text{ret}} \approx m_{\theta} d_{\text{BA}} \approx 0.2 \times 8 \text{ mm} \approx 1.6 \text{ mm}. \quad (3.19)$$

With a 1 inch focusing lens ($D \gg d_{\text{ret}}$), one has $d_{\text{eff}} \approx d_{\text{ret}}$, and the pinhole diameter can be chosen as

$$d_{\text{pinhole}} = n d_{\text{Airy}}, \quad (3.20)$$

where n is the pinhole size in Airy units. For $\lambda = 980 \text{ nm}$ and $d_{\text{ret}} \approx 1.2 \text{ mm}$ this yields

$$d_{\text{pinhole}} \approx n \frac{2.44 \lambda f}{d_{\text{ret}}} \approx n (2.0 \times 10^{-3}) f. \quad (3.21)$$

In this prototype, $n = 1.5$ was selected to remain close to conventional RCM choices ($n \approx 1$) while modestly enlarging the pinhole for improved SNR and reduced speckle sensitivity. This led to selecting a 1 inch achromatic doublet with $f = 35 \text{ mm}$ and a pinhole (Appendix A.8) of diameter

$$d_{\text{pinhole}} \approx 1.5 \times (2.0 \times 10^{-3}) \times 35 \text{ mm} \approx 0.10 \text{ mm} \approx 100 \mu\text{m}. \quad (3.22)$$

The detector placed after the pinhole is the 712B-2-DC, a silicon avalanche photodiode (APD) module from Analog Modules, with sensitive area diameter $\varnothing 0.5 \text{ mm}$, high transimpedance gain of the order of 1 MV/A or equivalently of $100 \text{ V}/\mu\text{W}$, and bandwidth up to $\sim 60 \text{ MHz}$ (-3 dB) (Appendix A.9). Although the peak responsivity of silicon occurs near $\sim 830 \text{ nm}$, operation at 980 nm remains feasible due to the high gain and bandwidth and a low noise (of the order of $\text{fW}/\sqrt{\text{Hz}}$). Using a conservative responsivity reduction factor of ~ 5 at 980 nm with respect to the peak, the output voltage may be estimated as

$$V_{\text{out}} \approx \left(\frac{100 \text{ V}/\mu\text{W}}{5} \right) P_{\text{in}} \approx (20 \text{ V}/\mu\text{W}) P_{\text{in}}. \quad (3.23)$$

For a representative sample illumination of ~ 10 mW and a confocal collection efficiency $\sim 10^{-6}$ (hence $P_{\text{in}} \sim 10$ nW), one obtains

$$V_{\text{out}} \sim 20 \text{ V}/\mu\text{W} \times 10 \text{ nW} \approx 200 \text{ mV}, \quad (3.24)$$

confirming adequate detection margin for subsequent digitization. Finally, to satisfy the axial scan requirement mentioned in Section 3.1, axial translation of the objective is implemented using the motorized linear stage LSA10A-T4A (Appendix A.10) with its dedicated controller X-MCC1 / KX13BF (Appendix A.11), both from Zaber. The stage provides a 10 mm travel range, a maximum speed up to 14 mm/s, fine velocity control down to 1.5×10^{-5} mm/s, nominal resolution $\sim 0.0238 \mu\text{m}$, repeatability $< 1 \mu\text{m}$, backlash $< 5 \mu\text{m}$, and load capacity ~ 25 N, which is more than sufficient for translating the objective over the clinically useful axial scanning range targeted by the prototype of 0.5 mm. The overall setup design described in this Section with all the mentioned optical components is represented in Fig. 3.3.2.

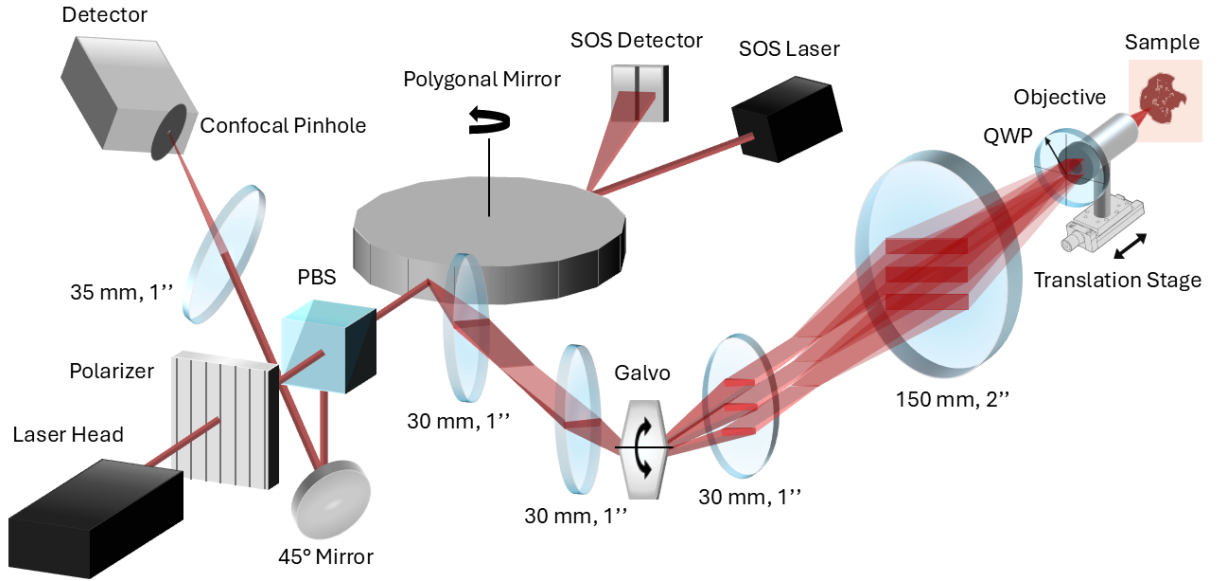


Figure 3.3.2: Overall optical and opto-mechanical layout of the RCM prototype.

Detailed mechanical integration and optical alignment are explained in Chapter 5, while scanner driving electronics, synchronization, control software, and the image reconstruction pipeline are presented in Chapter 4.

4 | Electronics and Control Software

4.1. Data Acquisition System and Scanners Control

This chapter presents the electronic architecture of the system, which enables reliable operation of the prototype and ensures correct interfacing among heterogeneous subsystems. The components requiring the greatest attention from an electronics standpoint—both in terms of power delivery and of driving/interfacing—are the scanning modules (rotating polygon mirror and galvanometric scanner), the photodetector, the optical synchronization subsystem used for image reconstruction, and the motorized translation stage hosting the objective. Since these components are sourced from different vendors, a dedicated electronic infrastructure is required to guarantee coordinated and synchronous operation. The analysis of the electronics begins with the central element that coordinates the entire setup, namely the Red Pitaya *STEMlab 125-14* DAQ board (Appendix A.12). The *STEMlab 125-14* is a compact platform based on a Xilinx Zynq system-on-chip integrating a dual-core ARM Cortex-A9 processor and an FPGA fabric; this heterogeneous architecture is particularly advantageous in high-speed scanning and microscopy systems because it allows a clear separation of tasks, with the processor handling high-level control (configuration, communication, measurement logic) and the FPGA implementing deterministic real-time functions (e.g., waveform generation and low-jitter synchronization with fast acquisition). The board is connected to the host PC via Ethernet, which represents the primary interface for control and development. In this prototype the board runs a custom control software (Section 4.3) developed in C and executed on the embedded Linux environment running on the ARM processor, while the FPGA remains available for hardware-timed triggering/synchronization functions when required. All the electronics connections between the *STEMlab 125-14* and the other elements are shown in Fig. 4.1.1 and in Fig. 4.1.2. In this section, the most important connections for the correct functioning of the prototype are explained, but a more complete report of all the electronics can be found in Appendix B. From the I/O perspective, the *STEMlab 125-14* provides two high-speed analog inputs and two high-speed analog outputs (14-bit, 125 MS/s). The inputs are DC-coupled and offer selectable full-scale ranges of ± 1 V (LV) and ± 20 V (HV), with an input impedance of $1\text{ M}\Omega$ and a bandwidth specified up to ~ 60 MHz, which matches the bandwidth of the selected detector and is therefore suitable for direct acquisition of the detector voltage. The outputs are also DC-coupled and can generate signals in the ± 1 V range into $50\text{ }\Omega$ output impedance, with bandwidth of ~ 60 MHz. This is sufficient in frequency content for the scanners driving at the kHz (polygon) and sub-kHz (galvo) scale, but it is generally insufficient in amplitude to directly meet the requirements of the scanners hardware modules. For this reason, an auxiliary conditioning/amplification board is introduced downstream of the *STEMlab* outputs, as discussed

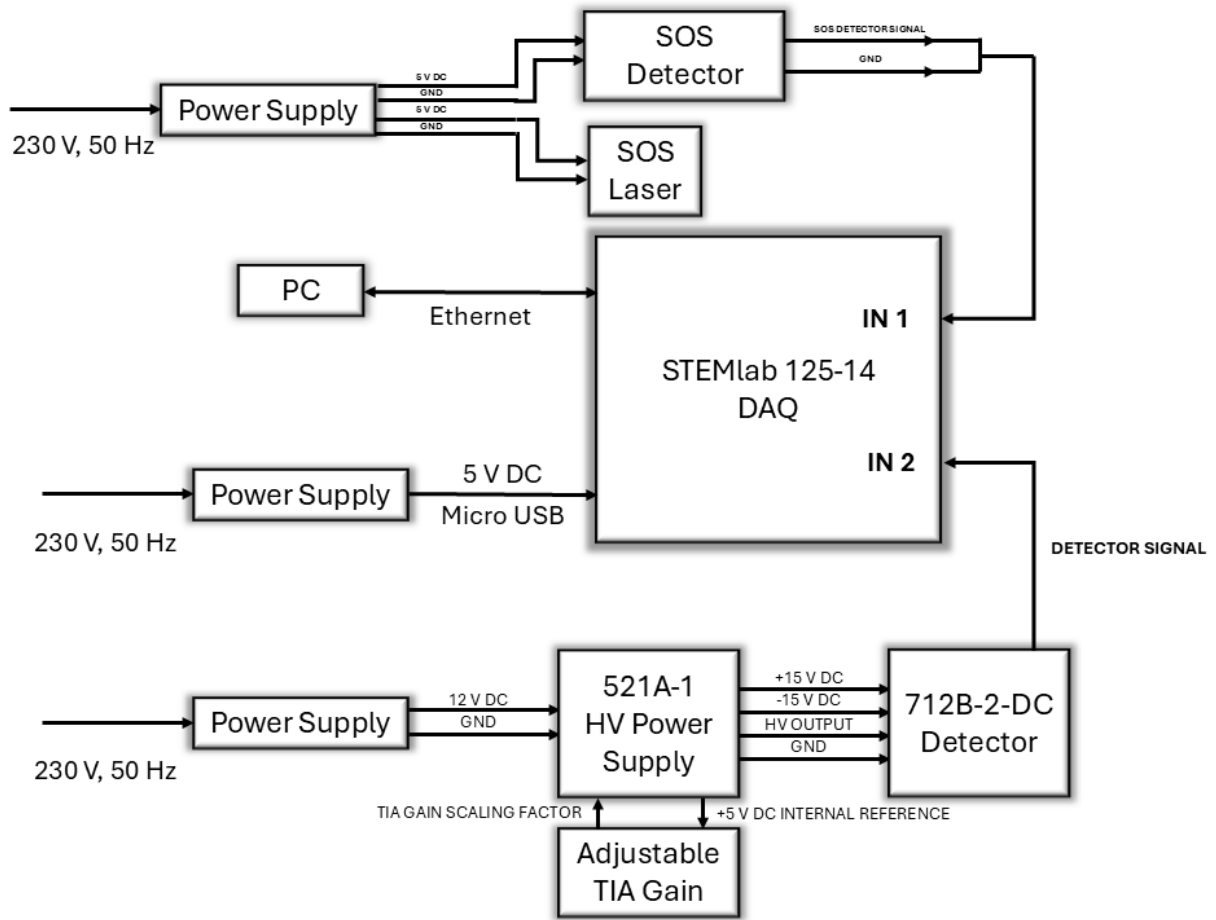


Figure 4.1.1: Scheme of the electronics connections on the STEMLab 125-14 radio-frequency analog input channels.

later in this section. In addition to the SMA high-speed analog channels, the STEMLab exposes two expansion connectors with digital I/O lines, which can be leveraged for trigger/synchronization and interfacing with external electronics. The first input is the detector output: the light backscattered by the sample is converted by the detector into an analog voltage, which is routed to one of the STEMLab analog inputs via a BNC cable, sampled by the on-board ADC, and streamed to the PC via Ethernet for logging and subsequent processing by the dedicated software (Section 4.3). The second input originates from the SOS (*Start Of Scan*) subsystem, responsible for synchronizing the rotating polygon mirror with the galvanometric scanner. The SOS photodetector converts the illumination of the SOS laser into a short electrical pulse each time the SOS beam crosses its active area. This pulse is then acquired by the second STEMLab analog input and used by the control software as an event marker corresponding to the start/end of each horizontal scan line operated by the polygon scanner. As detailed in Section 4.2, this timing reference allows the reconstruction algorithm to correctly map the acquired

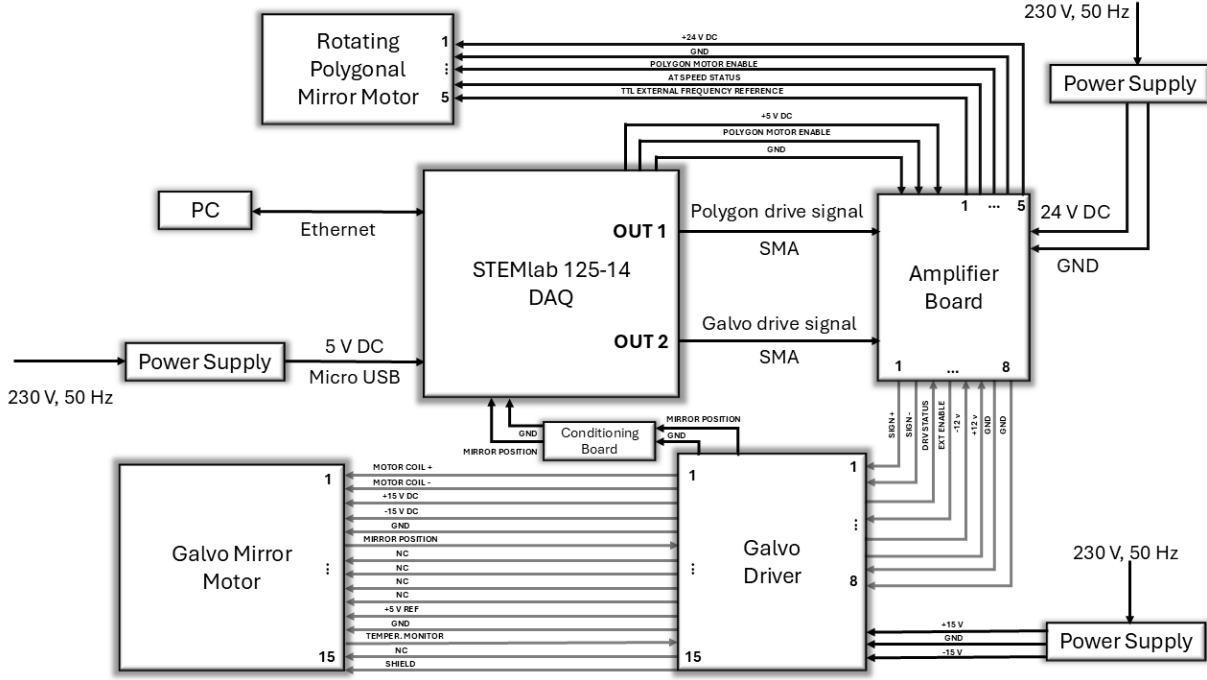


Figure 4.1.2: Scheme of the electronics connections on the STEMLab 125-14 radio-frequency analog output channels and slow I/O extension connectors pins.

data into a 1000×1000 pixel matrix and thereby generate a meaningful image. Although the STEMLab DACs offer sufficient timing resolution and low latency for scanner driving, their native output amplitude is limited to ± 1 V, which is not directly compatible with the scanner-driver requirements: the polygon scanner requires a TTL reference signal with logic levels 0–5 V, while the galvo driver expects a bipolar analog command that can reach up to ± 10 V depending on the desired scan amplitude. Consequently, the STEMLab analog outputs are routed to a dedicated conditioning board rather than directly to the scanners. The board is powered using the ± 12 V auxiliary outputs available from the galvo driver, which supply the operational-amplifier stages used to condition the STEMLab signals. The galvo command path employs two cascaded gain stages ($G_1 = 2$ followed by $G_2 = 5$), resulting in an overall gain $G = G_1 G_2 = 10$:

$$V_{\text{commandgalvo}} = 10 V_{\text{STEMLab}}, \quad (4.1)$$

thereby mapping the STEMLab output range ± 1 V to ± 10 V at the galvo-driver input. The same ± 12 V rails are also used to power the polygon reference-signal conditioning stage, which applies a gain $G = 5$ to convert the 0–1 V square wave generated by the

STEMlab into a 0–5 V TTL-compatible reference at the polygon input:

$$V_{\text{commandpolygon}} = 5 V_{\text{STEMlab}}. \quad (4.2)$$

This frequency reference input (TTL square wave at 50% duty cycle) sets the desired rotation speed according to

$$\text{RPM} = 15 f_{\text{ref}}, \quad (4.3)$$

where f_{ref} is the reference frequency in Hz. For example, operation at the maximum speed of 45,000 RPM requires $f_{\text{ref}} = 3$ kHz, whereas operation at the minimum recommended speed to protect the air bearing (25,000 RPM) requires $f_{\text{ref}} \approx 1.67$ kHz. The polygon connector on the conditioning board also routes the external 24 VDC motor supply, the enable line, and the speed-lock open-collector output. In addition, a 3-pin header on the board receives digital signals from the STEMlab expansion I/O connectors: two static lines at +5 V and ground are used for status LED power supply on the conditioning board, while a 3.3 V control line is used to force the polygon enable input to the active LOW state, so that the motor is enabled as soon as the STEMlab is powered. The input signal coming from the GVS001 driver to the STEMlab extension connector is treated in the next section dedicated to the scanners synchronization, as it brings the signal containing information about the galvanometric mirror position for correct frame reconstruction. The axial scanning functionality is implemented via the Zaber LSA10A-T4A motorized linear stage driven by the Zaber X-MCC1-KX13BF controller. From an electronics and interfacing standpoint, the connections between these two elements are shown in Fig. 4.1.3. In the setup the controller is connected to the host PC via USB

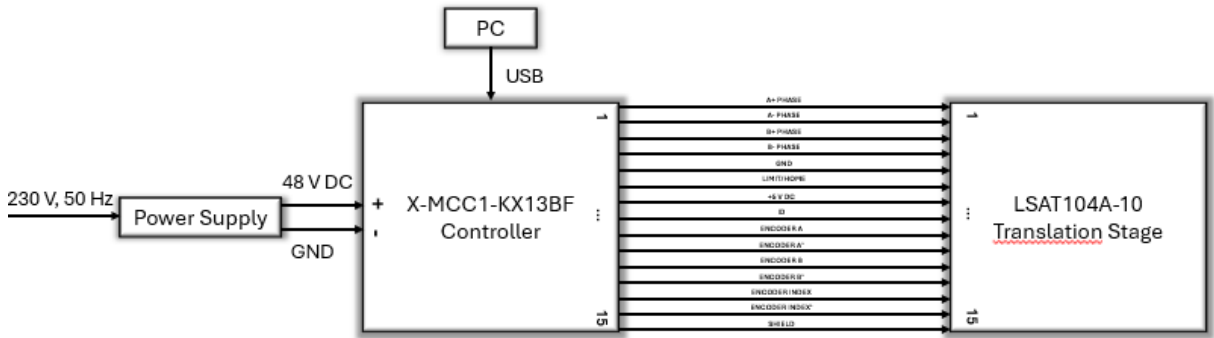


Figure 4.1.3: Scheme of the electronics connections between the motorized translation stage and its controller.

and integrated into the dedicated Zaber software so that objective position commands, velocity settings, and state queries can be issued programmatically and synchronized with acquisition routines when needed. Finally, the last two elements to detail electronically

are the laser head and the detector. Proper powering of the Analog Modules 712B-2 detector requires both a low-voltage supply for the module electronics (transimpedance amplifier) and a high-voltage reverse-bias for the APD. In this prototype, a programmable high-voltage bias module (Analog Modules 521A-1) is used to generate the required bias in the $\sim 180\text{--}250\text{ V}$ range. The HV module is itself powered from a $+12\text{--}+15\text{ VDC}$ source and provides a regulated output (up to $\sim +300\text{ V}$) programmable through a $0\text{--}5\text{ V}$ control input referenced to the system ground, enabling safe and repeatable optimization of the APD operating point, via TIA gain regulation. Finally, the MDL-III-980L laser head is interfaced with the PSU-III-LED Controller through a 15-pin connector that integrates power supply, thermal stabilization, and safety monitoring (connections represented in Fig. 5.1.1). High-current lines (LD+/-) drive the laser diode, while a dual-channel feedback loop, comprising TEC (Thermoelectric Cooler) modules and NTC thermistors, ensures precise closed-loop temperature control for both the diode and the crystal. The interface is completed by a hardware Interlock safety circuit and dedicated grounding lines to maintain signal integrity and prevent thermal drift during operation. As already mentioned, all the electronics connections between the various elements of the

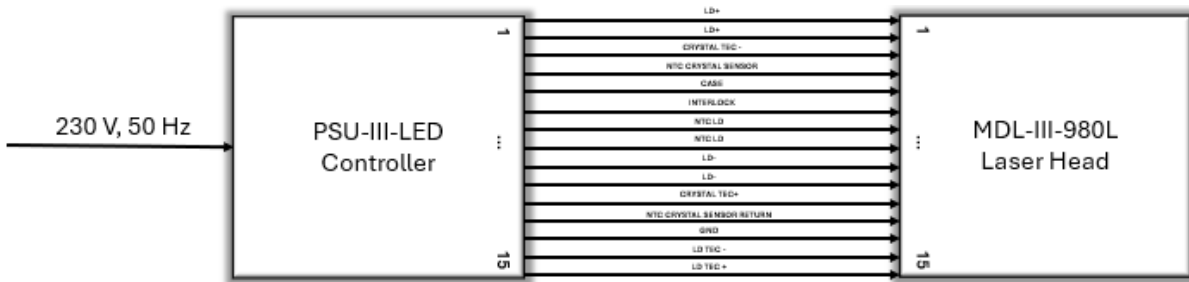


Figure 4.1.4: Scheme of the connections between the laser head and its controller.

setup are further explained in Appendix B.

4.2. Synchronization of the Scanning Hardware Modules

In a scanning confocal microscopy system, the image is not acquired as a single two-dimensional full-field frame. Rather, it is synthesized progressively by sampling in time the detected intensity while the focused spot scans the specimen. In this setup, horizontal scanning is produced by the rotating polygon mirror (fast axis), whereas the vertical coordinate is advanced by the galvanometric mirror (slow axis). For an unambiguous and geometrically correct reconstruction, each detector sample must be associated uniquely with the instantaneous scan position and mapped into the correct pixel of the image. Any timing ambiguity or mis-registration directly translates into geometric distortion, line-to-line misalignment, and visible artifacts. This section describes both the *Start Of Scan* (SOS) subsystem, which provides optical synchronization for the horizontal axis and the vertical reference for the frame reconstruction. This refers to the method by which the STEMLab communicates to the image reconstruction algorithm running on the PC which acquired lines correspond to the beginning of a frame and which correspond to its end, thereby defining a vertical synchronization with the position of the galvanometric mirror during the scan. The SOS subsystem consists of two elements (shown in Fig. 4.2.1). A

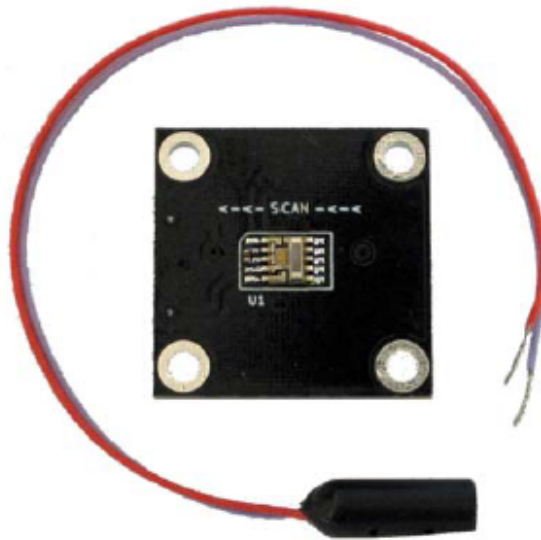


Figure 4.2.1: SOS system: Detector and Laser diode.

low-power diode laser ($\lambda = 650 \text{ nm}$, $P < 1 \text{ mW}$) powered from 5 VDC, and a compact photodetector whose active area is sensitive to the SOS laser wavelength and whose 4-pin connector provides power and output signal interfacing (more information can be found in

Appendix A.13). The SOS laser and detector are mechanically fixed around the polygon scanner and aligned such that the SOS beam is reflected by a facet into the detector only for a specific angular configuration of the system. As the polygon rotates, the reflected SOS beam sweeps linearly in time, analogously to the main illumination beam, and the geometric condition defining the start of scan event repeats once per facet. For a polygon with $N_f = 16$ facets, this condition occurs every $1/16$ of a revolution, thus producing one SOS event per scan line. The corresponding line scan rate and line period are

$$f_{\text{line}} = \frac{\text{RPM}}{60} N_f, \quad T_{\text{line}} = \frac{1}{f_{\text{line}}}. \quad (4.4)$$

The SOS detector generates a TTL-compatible digital pulse in correspondence of the start-of-scan condition. During normal operation, the SOS line remains HIGH and changes to LOW when the laser beam crosses the detector active area. In particular, the signal is *active-low* (LOW identifies the SOS condition) and exhibits a fast falling transition (< 7 ns). Operationally, the timing reference is taken as the HIGH→LOW edge (SOS event) and is acquired by the STEMLab (by sampling the signal coming from the SOS detector and detecting falls), where it is digitized and sent to the Pc as described in Section(4.3). Under the assumption of constant polygon rotational speed and linear scan within the selected usable window of each facet, the elapsed time $\Delta t = t - t_{\text{SOS}}$ provides a monotonic mapping to the fast-axis coordinate and, according to the STEMLab ADCs sampling rate, to the image-column index. The SOS manual further emphasizes that, when feasible, using the same polygon facet for both the SOS beam and the main illumination beam is advantageous, as it reduces facet-to-facet timing offsets and improves line-to-line registration, ultimately enhancing reconstruction fidelity and minimizing residual geometric jitter. However, this was not feasible in this prototype due to space constraints related to the optical components. Having addressed horizontal synchronization, the vertical one is now discussed. In the absence of information on the instantaneous position of the galvanometric mirror during acquisition, an SOS-only scheme would merely allow the PC to reconstruct individual lines and stack them into blocks of 1000 (recalling that the target image size is 1000×1000 pixels), without any guarantee that the line displayed as the first (top) row of a frame actually corresponds to data acquired when the galvanometric mirror is at its maximum scan angle. Since the galvanometric mirror is much slower than the rotating polygon mirror, its angular position can be regarded as effectively constant over the acquisition time of a single line. This missing information is therefore provided to the STEMLab via one of its digital input pins on the extension connectors from the galvanometric mirror driver. Among the 15 pins connecting the galvanometer motor to the driver (specifically pin #6 in Appendix B.5), the motor outputs a real-time voltage

signal proportional to the mechanical deflection angle with respect to the rest position, referenced to 0 V input voltage and scaled by a fixed factor of 0.5 V/deg:

$$V_{\text{pos}}(t) = (0.5 \text{ V/deg}) \theta_{\text{mech}}(t) \quad (4.5)$$

The mechanical scan angle $\theta_{\text{mech}}(t)$ is determined by the following relationship:

$$\theta_{\text{mech}}(t) = \frac{V_{\text{com}}(t)}{0.5 \text{ V/deg}} \quad (4.6)$$

In practice, the scaling factor linking the input voltage to the mechanical scanning angle can be configured via a jumper on the galvanometric mirror driver to assume values of 0.5 V/deg, 0.8 V/deg, or 1 V/deg. In this prototype, a scaling factor of 0.5 V/deg was selected, resulting in the following identity:

$$V_{\text{pos}}(t) = 0.5 \text{ V/deg} \cdot \frac{V_{\text{com}}(t)}{0.5 \text{ V/deg}} = V_{\text{com}}(t) \quad (4.7)$$

According to the calculations reported in Section 3.2, the galvanometric mirror must perform a scan of $\pm 14.6^\circ$ (optical), which corresponds to a mechanical scan of:

$$\pm \frac{\theta_{\text{opt}}}{2} = \pm \frac{14.6^\circ}{2} = \pm 7.3^\circ_{\text{mech}} \quad (4.8)$$

Given the aforementioned scaling factor, this angular range is obtained with a driving voltage signal oscillating within $7.3^\circ \cdot 0.5 \text{ V/deg} = \pm 3.65 \text{ V}$. Consequently, $V_{\text{pos}}(t)$ will also oscillate within the same interval. However, the STEMLab inputs located on the extension connectors accept a dynamic range between 0 and 3.5 V. A signal oscillating at $\pm 3.65 \text{ V}$ would therefore be clipped, introducing non-linear effects that are difficult to interpret for the frame reconstruction software. Consequently, the $V_{\text{pos}}(t)$ signal is first routed to a conditioning board that rescales it to the 0–3.5 V range. Through this process, the negative portion of $V_{\text{pos}}(t)$ is ignored, while its positive portion, including the peaks at 3.65 V, is sent to the STEMLab. The board can sample the waveform correctly since the signal oscillates at a frequency in the order of 10 Hz, well within the 250 kHz bandwidth of the extension connector input channels, allowing for the frame reconstruction processing described in Section 4.4. It is now necessary to define the relationship between the scanner driving frequencies, the STEMLab sampling rate, the detector bandwidth, and the resulting frame rate. To achieve:

$$N_r = 1000 \text{ pixels/line} \quad 1 \text{ pixel} = 1 \text{ sample} \quad (4.9)$$

the following condition must be met:

$$N_r = T_{\text{line}} \cdot f_{\text{s,eff}} = 1000 \quad (4.10)$$

where $T_{\text{line}} = 1/f_{\text{line}}$ is the time required for the rotating mirror to scan a single line and $f_{\text{s,eff}}$ is the effective sampling frequency of the STEMLab ADC. This implies:

$$N_r = \frac{1}{f_{\text{line}}} \cdot \left(\frac{f_{\text{s,max}}}{D} \right) \quad (4.11)$$

where $f_{\text{s,max}} = 125 \text{ MS/s}$ from datasheet (Appendix A.12) and D is the decimation factor, a scaling factor that allows to reduce the ADC sampling frequency to an integer sub-multiple of the maximum frequency. Substituting the physical parameters of the polygon mirror:

$$N_r = \left(\frac{60}{\text{RPM} \cdot 16} \right) \cdot \left(\frac{f_{\text{s,max}}}{D} \right) \quad (4.12)$$

$$N_r = \left(\frac{60}{15 \cdot f_{\text{square}} \cdot 16} \right) \cdot \left(\frac{f_{\text{s,max}}}{D} \right) \quad (4.13)$$

where f_{square} is the reference square wave frequency for the rotating mirror from equation 4.3. This leads to the relation:

$$f_{\text{square}} \cdot D = 31250 \text{ Hz} \quad (4.14)$$

Considering the constraints on D and the operational speed limits of the rotating mirror motor (between 25,000 and 45,000 RPM, corresponding to f_{square} between 1666.67 Hz and 3000 Hz), the following parameters were chosen:

$$D = 16, \quad f_{\text{square}} = 1953.125 \text{ Hz} \quad (4.15)$$

This results in a rotation speed of:

$$\text{RPM} = 29296.875 \rightarrow f_{\text{line}} = 7.8125 \text{ kHz} \quad (4.16)$$

Therefore, for a 1000×1000 pixel image, the frame rate is:

$$\text{fps} = \frac{7812.5 \text{ lines/s}}{1000 \text{ lines/frame}} = 7.8125 \text{ fps} \quad (4.17)$$

Since the galvanometric mirror is driven by a ramp that scans from top to bottom, where each ramp corresponds to one frame, the galvanometric mirror frequency is set as

$$f_{\text{galvo}} = \text{fps} = 7.8125 \text{ Hz} \quad (4.18)$$

The detector bandwidth, being in the order of tens of MHz, does not constitute a limiting factor for the acquisition. Under these conditions, every second the polygonal mirror scans 7812.5 lines, the galvanometric mirror completes 7.8125 vertical scans, and the STEMLab samples:

$$\frac{125 \text{ MS/s}}{16} = 7.8125 \text{ MS} \quad (4.19)$$

With a ratio of 1 MS/frame, this corresponds exactly to 7.8125 frames per second. In the next section, the system control software will be detailed and analyzed in depth.

4.3. Control Software Architecture

In this section, the control software is described in detail. The analysis clarifies the central role of the STEMLab in the system operation and its interaction with the host Pc. The STEMLab acts as the central node, bridging analog signals to the digital domain via its 14-bit ADCs and, at the same time, generating the drive waveforms for the scanners through its DACs. The software architecture is distributed across three layers: (i) the firmware logic in the FPGA, (ii) a multi-threaded C application running on the STEMLab ARM CPU, and (iii) a Python-based TCP server on the host Pc. The core of the embedded system is the multi-threaded C application (`RedPitaya2.c`) designed for high-performance concurrency. The software utilizes the `pthread` library to implement a producer-consumer pattern, ensuring that data acquisition from the hardware and network transmission to the Pc occur in parallel. The STEMLab utilizes a Zynq System-on-Chip (SoC) that combines a FPGA (Field-Programmable Gate Array) with an ARM CPU (Advanced RISC Machine). While the FPGA handles the real-time processing of the 125 MS/s ADC streams, the ARM CPU, running an embedded Linux, executes the high-level logic for data handling and TCP/IP communication. The FPGA is a specialized semiconductor device consisting of a matrix of configurable logic blocks that can be programmed to perform complex hardware-level tasks at extreme speeds. While FPGAs are ideal for the real-time processing of high-frequency signals, such as the 125 MS/s ADC streams, they possess significant internal memory constraints. Specifically, the FPGA's local Block RAM (BRAM) - a small, ultra-fast memory embedded directly into the silicon logic - is limited to a few kilobytes. For a high-resolution imaging system requiring $N_r = 1000$ pixels per line and 1000 lines per frame, a single acquisition cycle generates 10^6 samples per channel, which would immediately saturate the BRAM. To overcome the internal memory constraints of the FPGA, the system operates in Deep Memory Mode. In this configuration, the FPGA bypasses its local registers and utilizes the AXI (Advanced eXtension Interface) bus - a high-performance communication "highway" - to write data directly into the system's DDR RAM (Double Data Rate RAM, the larger external system memory, which is significantly larger but slightly slower than the BRAM) via DMA (Direct Memory Access). DMA is a critical mechanism that allows hardware modules to transfer data directly to the RAM without involving the main CPU. This bypass prevents the CPU from becoming a bottleneck, ensuring that no samples are lost during high-speed streaming. This architecture effectively transforms the system RAM into a massive circular buffer, enabling the acquisition of large-scale datasets. A circular buffer is a data structure that uses a single, fixed-size buffer as if it were connected end-to-end. In this context, it allows the FPGA to continuously write new data over the oldest samples only.

after they have been successfully transmitted, ensuring a seamless data stream. All the code that now will be explained in depth can be found in Appendix C. On the host Pc, the Python script `server_dual4b.py`, executed from the Command Prompt (`cmd`), creates a TCP server by opening a socket, binding to an IP address and port (for the Pc used in the setup: `169.254.95.215:1234`), listening (command `listen(1)`), and waiting for an incoming connection (`accept()`). In this configuration, the PC remains passive until the STEMLab initiates contact via Ethernet. On the STEMLab side, the C software (`RedPitaya2.c`) is executed by the embedded Linux running on the board's ARM CPU. It is launched remotely from the PC via Windows PowerShell through an Ethernet SSH connection. This program acts as a TCP client: it creates a socket and establishes the data channel using `connect()` toward the PC IP–port pair. Once the connection is open, the Python server enters a continuous loop in which it robustly and continuously receives the stream: it initializes an accumulator as an empty byte sequence using `recv_data = b''` and enters a second loop that repeatedly calls `recv()` until exactly a fixed-size payload (`bufsize`) has been received. This explicitly handles the fact that a single `recv()` call may return only a partial portion of the requested block. As soon as the complete block is available, the program deterministically splits it into two halves (first half = channel 1, second half = channel 2) and appends the corresponding bytes to disk, generating `adc.npy` for the signal acquired from the main detector and `sos.npy` for the signal from the SOS detector, as well as `both.npy` containing the full packet. In this way, the recorded data files grow progressively, block after block. This splitting follows the same convention used to write the data into the transmission buffer, as described later in this section. It should be emphasized that, despite the `.npy` extension of the generated files, at this stage the write operation is a raw binary *append* and not a standard NumPy `.npy` format (which would include a header). Therefore, the subsequent readout performed by the image reconstruction program will handle this as described in Section 4.4. Before acquisition, the C program running on the STEMLab configures the analog outputs by programming the DACs through the vendor-provided Red Pitaya library (functions prefixed by `rp`, e.g., `rp_Init()` and the `rp_Gen*` calls). The first analog output channel (CH1) drives the rotating polygon mirror and is therefore configured as a square wave at the frequency required to achieve the desired rotation speed, with native amplitude 0–1 V. As explained in Section 4.1, the auxiliary conditioning board containing amplification stages increases this range to 0–5 V, as required by the polygon motor driver. The second channel (CH2) drives the galvanometric mirror and is configured as a ramp (*ramp-down*/sawtooth) at the desired frequency to implement the slow scan axis. Conceptually, these signals are “programmed” by the C code on the STEMLab, but they are physically generated by the board hardware blocks (DACs and associated logic). The C code running on the STEMLab ARM

CPU also configures the acquisition hardware parameters (effective sampling frequency, trigger, addresses, buffer sizes, etc.) and, once the FPGA logic has completed writing samples into DDR, transfers the data to the PC by reading contiguous memory blocks. More specifically, the C code retrieves the DDR region reserved for acquisition using the Red Pitaya library call `rp_AcqAxiGetMemoryRegion(&mem_start, &mem_size)` and logically splits it into two independent halves, one for CH1 and one for CH2 (`mem_mid = mem_start + mem_size/2`), thus enabling simultaneous acquisition from both analog inputs. The program then sets the decimation factor 16 via `rp_AcqAxiSetDecimationFactor(16)`, yielding an effective sampling frequency

$$f_s = \frac{125 \text{ MS/s}}{16} = 7.8125 \text{ MS/s},$$

which matches the scan frequency of the scanners. A trigger delay consistent with the buffer size is also configured, such that after the trigger event the hardware records a buffer of fixed, known length (i.e., a constant number of samples). Efficient management of the ADC data stream is implemented using a DDR ring buffer. A buffer size per channel is defined by the variable `BUFFER_HALF_SAMPLES`, initialized to

$$\text{BUFFER_HALF_SAMPLES} = 1000 \cdot 1000 = 10^6,$$

Since each ADC sample is represented with 16 bits (2 bytes), this corresponds to a per-channel buffer size of

$$1,000,000 \times 2 \text{ B} = 2,000,000 \text{ B} = 2 \text{ MiB}.$$

This block size is sufficiently large to reduce management overhead (fewer acquisition and transfer iterations, fewer inter-thread synchronizations, and fewer TCP sends per unit time), while remaining small enough to avoid excessive latency and DDR usage. Moreover, by mapping a contiguous DDR window of size `DMA_MAP_SIZE = 128 MiB` (i.e., the region made accessible to the process via `mmap()`) and splitting it into two halves (one for CH1 and one for CH2, i.e., 64 MiB per channel), a 2 MiB buffer yields an integer number of ring-buffer slots:

$$\frac{64 \text{ MiB}}{2 \text{ MiB}} = 32,$$

which motivates the initialization of `NUM_BUFFERS` in the code. This correspondence avoids fragmentation and unused memory and enables cyclic buffer management using

offsets computed as

$$\text{offset}[i] = i \cdot \text{BUFFER_HALF_SAMPLES} \cdot \text{sizeof}(\text{uint16_t}).$$

For each index i , CH1 samples are written in DDR at `mem\_start+offset[i]`, and CH2 samples at `mem\_mid+offset[i]`. To prevent overwriting and to maintain a continuous pipeline between acquisition and transmission, the software uses two semaphores per buffer: `sem\_dma\_done[i]` initialized to 1 (buffer i is “free” and can be filled by the hardware) and `sem\_adc\_done[i]` initialized to 0 (buffer i is not yet ready to be read/sent). In addition, the program allocates three process-local buffers: `buf` (local copy of CH1 samples), `buf2` (local copy of CH2 samples), and `combo_buf` (concatenation CH1+CH2, ~ 8 MiB), which matches exactly the payload expected by the PC. Each packet begins with a custom 84-byte metadata header (`mkr_header_t`), which contains a “magic number” for packet validation, the circular buffer index, and an array of up to 16 frame-start markers captured in real-time. This header is immediately followed by the raw payload, where the 1,000,000 samples of Channel 1 (reflection signal) and Channel 2 (SOS signal) are concatenated sequentially before being dispatched via the TCP/IP socket. The correspondence between “first half/second half” of the received block and the two signals (main detector and SOS) is defined by a packaging convention: the STEMLab program always concatenates samples in the same order (first CH1, then CH2), and the Python script applies the same convention by splitting the payload in half and saving the first portion to `adc.npy` and the second to `sos.npy`. This clarifies the generation mechanism of `adc.npy` and `sos.npy` mentioned earlier. The acquisition and transmission chain is implemented with due threads. The ADC thread prepares and starts acquisition and, for each buffer i , begins by waiting on `sem_wait(&sem_dma_done[i])`, preventing slot i of the DDR ring buffer from being overwritten with new data before the samples it contains have been read and sent to the PC by the transmission thread. The ADC thread then configures in the FPGA the addresses and size of the next buffer to acquire using the Red Pitaya library function `rp_AcqAxiSetBufferSamples(RP_CH_1, mem_start + offset[i], BUFFER_HALF_SAMPLES)` for the first channel and similarly for the second channel. Subsequently, it enables AXI acquisition on both channels (`rp_AcqAxiEnable(..., true)`), starts acquisition (`rp_AcqStart()`), manages triggering, and waits for hardware events via polling. The ADC thread waits until the DDR buffers are full on both channels by querying the fill state (`rp_AcqAxiGetBufferFillState`), then stops acquisition (`rp_AcqStop()`) and unblocks the next thread via `sem_post()`. The DMA/SEND thread runs in parallel and, for each iteration i , waits on `sem_wait()`, then reads the newly acquired samples from DDR, copies them into `buf` and `buf2`, builds

`combo_buf` by concatenating first all CH1 samples and then all CH2 samples, and finally sends the full block over the TCP socket to the Python server. Once transmission is complete, it signals that the DDR buffer can be reused via `sem_post(&sem_dma_done[i])`, thereby realizing a continuous pipeline

acquisition $\rightarrow$ copy $\rightarrow$ send

without collisions. Direct DDR readout is implemented in `dma_dma.c` and `dma_dma.h`, which provide a low-level interface to physical memory. The function `dma_init()` opens `/dev/mem` — the device file that allows access to physical memory — and maps, at application level (via `mmap`), a physical memory window starting at the base address `DMA_BASE_ADDR` with size `DMA_MAP_SIZE`. This operation returns a pointer (`dma_mem`) enabling direct access to the samples deposited by the FPGA. The function `dma_read(dest, absolute_addr, length)` computes the relative offset as

`absolute_addr - dma_offset_base`

and then performs a `memcpy` from the mapped region into the STEMLab process RAM buffer. This allows the DMA thread to quickly fetch the blocks associated with `mem_start + offset[i]` (CH1) and `mem_mid + offset[i]` (CH2) without relying on higher-level acquisition calls. In this way, synchronization in terms of “position along a scan line at a given instant” relies on the fact that the SOS system provides a repeatable time reference. In practice, the STEMLab FPGA does not reconstruct the x -coordinate pixel-by-pixel in real time. Instead, it produces samples with an implicit timestamp (the sample index, determined by the ADC clock and the decimation factor), while the SOS signal provides the anchor required to delimit scan lines. Each SOS pulse marks the start of a line, and the number of samples between two consecutive pulses defines the line duration (in samples). Knowing the effective sampling frequency f_s , each sample index n corresponds to a time

$$t = \frac{n}{f_s}.$$

Assuming constant polygon rotation speed, the time elapsed since the SOS event is proportional to the position along the fast axis. This enables post-processing selection of the useful sample window and remapping into the 1000 columns of the image matrix, while the slow axis (galvo) determines the line-to-line advance and therefore the vertical index, which will now be discussed. The acquisition of the signal containing the reference for the vertical synchronization also begins in the Acquisition Thread on the RedPitaya. While the FPGA is streaming millions of samples per second from the fast analog input

channels into the DDR RAM, the auxiliary signal on pin AI1 is sampled by the XADC (Xilinx Analog-to-Digital Converter), a dedicated hardware module integrated within the Zynq-7000 SoC. Unlike the high-speed ADCs, the XADC features a 12-bit resolution and operates at an aggregate sampling frequency of 1 MS/s (shared by all AI channels), which is more than sufficient for the galvanometric mirror position signal, oscillating at a frequency on the order of a few Hz to tens of Hz. In this system architecture, the XADC is controlled and monitored by the ARM CPU through C code. This allows the CPU to detect the galvanometric ramp reset and generate temporal markers without consuming programmable logic (FPGA) resources dedicated to the high-speed 125 MS/s data stream. The software uses the function `rp_AIpinGetValue` to continuously monitor the voltage of the mirror position signal which, as explained in Section 4.2, matches exactly the voltage ramp used to drive the mirror. When the voltage crosses a specific threshold (representing the “flyback” or reset of the mirror), the software identifies a “Frame Start” event. At the exact moment of detection, the code calls `rp_AcqAxiGetWritePointer`. This function returns the index of the sample currently being written by the FPGA relative to the start of the current buffer slot. For example, if the reset occurs exactly halfway through the i -th buffer, the local marker m might be 500,000. The local markers are not sent to the host PC alone. They are embedded into a custom 84-byte metadata header (`mkr_header_t`). The C code fills an array called `markers[16]` with the captured offsets and sets the `marker_count`. The transmission thread sends this header over the network immediately followed by the raw ADC payload (the 1,000,000 samples for CH1 and CH2). When the Python script `server_dual4b.py` receives the data, it must convert the local marker (relative to one buffer) into a global index (relative to the start of the entire experiment). To accomplish this, the script maintains a running counter called `global_sample_base`. Every time a new packet is successfully received, this counter is incremented by the number of samples per channel (e.g., 1,000,000). For each marker m found in the incoming header, the script computes the absolute position

$$I_{\text{global}} = \text{global_sample_base} + m.$$

The final step consists of saving these global indices to disk. The Python script uses

```
struct.pack("<Q", gi)
```

to convert the calculated global index into a 64-bit unsigned integer (`uint64`) in little-endian format. This 8-byte value is appended to `frame_markers.bin` and sent to the host PC together with `adc.npy` and `sos.npy`. The whole process described in this section is illustrated in Figure 4.3.1. For the sake of reproducibility, all the source code and

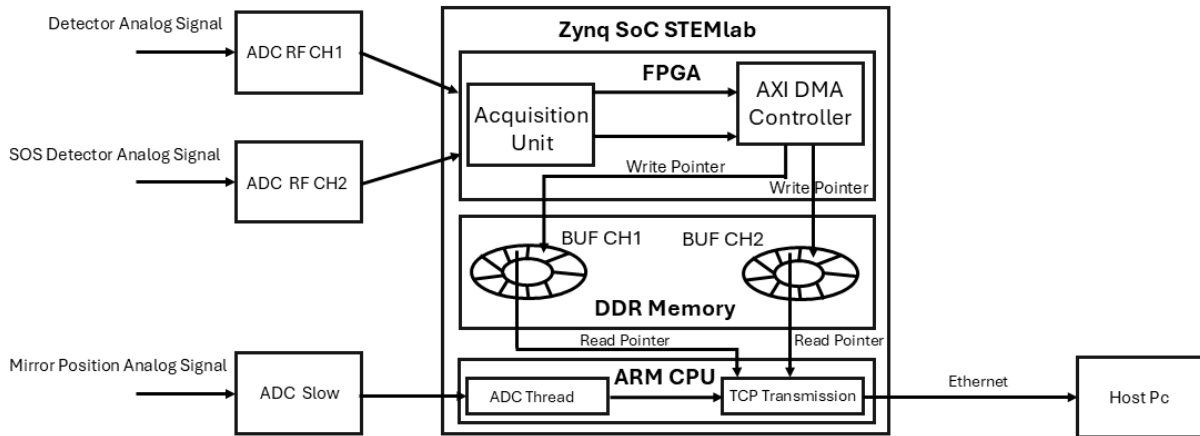


Figure 4.3.1: Data acquisition and transmission towards the host PC process scheme.

the documentation and guidelines required to operate the STEMLab-controlled prototype are available in Appendix C. In the next section, another software component will be discussed, namely the code responsible for data interpretation and image reconstruction on the PC.

4.4. Image Reconstruction Algorithm and Visualization GUI

The image reconstruction and visualization workflow was implemented in the Python script `Visualize.py`, which integrates both (i) the conversion of the raw acquisition streams into image frames and (ii) a graphical user interface (GUI) for inspection and verification. The program is designed around the specific output format produced on the host computer after the data acquisition, namely three files stored in the same folder: `adc.npy`, `sos.npy`, and `frame_markers.bin`. As already introduced, the `adc.npy` stream contains the detector samples acquired by the STEMLab ADC (stored as a contiguous 1-D array of 16-bit signed integers), whereas `sos.npy` is a synchronization waveform (also stored as a 16-bit stream) used to detect scan-line boundaries. The third file, `frame_markers.bin`, stores the starting sample indices of each frame as 64-bit unsigned integers. Consecutive markers thus define the temporal span of each image frame in sample units. The host-side acquisition stores these files by the acquisition software detailed in Section 4.3. They use the `.npy` extension, but they do not implement the standard NumPy `.npy` container (header plus metadata). Instead, they encode a contiguous 16-bit sample stream, equivalent to a one-dimensional array. For this reason, the visualization software performs an automatic input-normalization step and converts the streams to a uniform `.bin` representation prior to reconstruction. Concretely, the function dedicated to this task, `_looks_like_numpy_npy()`, checks the first bytes of a file for the NumPy magic header (`\x93NUMPY`); if the header is absent, the file is treated as a raw 16-bit stream. In that case, `convert_raw_u16_npy_to_bin()` reproduces the legacy `npy_to_bin_adc_sos.py` behaviour by reading the file with `np.fromfile(..., dtype=np.uint16)` and rewriting the identical byte stream to a `.bin` file. After loading the files, the `adc` and `sos` arrays are aligned by truncating both to the minimum available length, and the marker list is filtered to retain only indices strictly within the valid sample range. Reconstruction then proceeds by extracting a discrete set of scan-line boundaries from the SOS channel. The SOS processing is based on a thresholded plateau detector: `find_falls()` computes a boolean plateau array (`sos >= SOS_THR`), differentiates it with `np.diff`, and identifies falling edges as the sample indices where the plateau transitions from HIGH to LOW. These raw edges are then regularized by `clean_min_dist()`, which enforces a minimum separation `MIN_EDGE_DIST` between consecutive edges, rejecting spurious double transitions and possible high-frequency glitches in the SOS waveform. The resulting vector `falls` is the fundamental segmentation primitive used throughout the remainder of the algorithm: each interval between two consecutive falling edges corresponds to

one scan line in the image. Because the early part of an acquisition can be affected by transient scan conditions (e.g., polygon mirror accelerating towards the target speed), `Visualize.py` does not reconstruct frames from the beginning of the file by default. Instead, it searches for a stability region in the SOS edge spacing. The array `distances = diff(falls)` is scanned with a sliding window. The first window whose peak-to-peak jitter is below `JITTER_TOL` and whose median spacing is within `PERIOD_TOL` of the expected period (`EXPECTED_PERIOD` in samples terms) defines the stability onset. The corresponding sample index `stable_sample` is used to select the first frame marker to reconstruct (`start_frame_idx`), ensuring that the reconstruction starts only when the scan-line cadence is sufficiently stationary. A further global alignment step is then applied to compensate for a constant phase offset between frame markers and SOS line boundaries. The program measures the offset `phase_ds` by taking the first stable frame start index `fs0` and finding the nearest SOS falling edge around it. The SOS edge vector is shifted by `phase_ds = fs0 - edge_near`. After shifting, the edges are clipped to the valid domain and deduplicated via `np.unique`. This operation does not change the internal geometry of each frame, but it ensures consistent anchoring of the first reconstructed line relative to the frame boundary, thereby improving the repeatability of the image origin across datasets. Frame reconstruction is carried out by iterating over consecutive marker pairs (`fs`, `fe`). For each frame, the SOS falling edges that lie within [`fs`, `fe`] are obtained by binary search (`np.searchsorted`) and sliced from the global `falls` vector. The resulting per-frame edge list is then refined by the function `repair_edges()`, whose purpose is to mitigate occasional missing edges (which merge two physical lines into one segment) or extra edges (which split a line into two short segments). `repair_edges()` computes a robust period estimate (median of edge-to-edge differences) and compares it to the nominal expected period; if the estimate is implausible, it is replaced by the expected value. Each subsequent edge is accepted, rejected, or augmented by inserting synthetic edges when a gap exceeds `max_ratio` times the period, and by dropping edges when a gap is below `min_ratio` times the period. The number of inserted and deleted edges is propagated to the diagnostic flags and later recorded in the per-frame report, allowing the user to identify frames that required substantial correction. Once a consistent edge list is available, each consecutive edge pair (`a`, `b`) defines the slice `adc[a:b]`, which contains the raw samples of a single scan line. Because the number of samples per line can vary slightly (due to timing jitter, scanner non-idealities, or residual edge-detection errors), each line is resampled to a fixed horizontal pixel count `TARGET_W` using `resample_1d()`, implemented as linear interpolation via `np.interp`. The set of resampled lines is stacked into a 2-D array with height equal to the number of detected lines `rows_raw`. A second resampling stage enforces a fixed vertical dimension `TARGET_H`: `resample_rows()` per-

forms column-wise linear interpolation to map `rows_raw` onto `TARGET_H` rows. At this point, the frame is clipped to the ADC physical range (-8192 to 8191 , consistent with a signed 14-bit representation) and cast to `int16`. To address the recurring appearance of visually disturbing “glitched” horizontal lines, `Visualize.py` performs two complementary corrections. First, a seam-repair mechanism detects an anomalously large jump between the medians of two consecutive reconstructed lines. The signed and absolute differences of row medians are evaluated; if the maximum jump exceeds the threshold `ROW_STEP_REPAIR_THRESH`, the most likely spurious row is replaced by the mean of its two neighbours. This targets the common case in which a missed or duplicated SOS edge causes one line to be assembled from mismatched samples. Second, a more global and robust “deglitch” step is applied by `deglitch_rows_mad()`. Here, the median absolute difference between consecutive rows is computed, and a robust z-score is formed using the median absolute deviation (MAD). Rows whose associated z-score exceeds a threshold are classified as outliers and replaced by the average of adjacent rows. A fail-safe condition prevents the correction from being applied if an unreasonably large fraction of rows is marked as outlier, reducing the risk of artefacts in heavily corrupted frames. The combined effect of these two procedures is a substantial reduction of isolated defective lines while preserving genuine high-contrast edges and texture in the specimen. In addition to vertical consistency, the program includes an automatic correction for a systematic horizontal phase shift that may arise when the mapping between SOS edges and the beginning of the ADC pixel stream is not perfectly aligned due to the different incidence positions of the main beam and of the SOS beam on the respective polygonal mirror facets. The mechanism is enabled by `AUTO_XSHIFT` and estimates a global circular shift in pixel units by analysing the intensity profile of the first stable frames. For each candidate frame, the median intensity profile along x is correlated (in the Fourier domain) with a centre-versus-border mask, and the shift that maximises the correlation peak is retained. Candidate estimates are accepted only if the profile exhibits sufficient contrast (quantified by a percentile-based strength metric), and the final global shift is obtained as the median of a small set of accepted candidates. Frames are buffered until the shift is determined and are then written after applying `np.roll(..., axis=1)`. This strategy avoids locking onto low-information frames and reduces the probability of phase-induced “split” light patterns in the reconstructed images, based on the knowledge of the expected laser spot shape on the detector. Each reconstructed frame is saved to disk in two formats. The primary output is a 16-bit TIFF file (`frame_XXX.tif`) written by `save_tiff_16()`, which maps the signed 14-bit range to the full unsigned 16-bit domain by offsetting by 8192 and left-shifting by 2 bits. In parallel, the same frame is stored as a NumPy array (`frame_XXX.npy`) to preserve the signed intensity representation for

subsequent eventual quantitative processing. Finally, the algorithm generates a diagnostic report `frame_report.tsv` containing per-frame metadata, including frame boundaries (`fs`, `fe`), phase offset, applied horizontal shift, the number of raw rows, resampling symmetry metrics, and a set of flags documenting any corrective action (edge repair, seam replacement, MAD deglitching). This report provides traceability and supports systematic tuning of the reconstruction parameters when required. The reconstruction pipeline is exposed to the user through a tab-based GUI implemented with `tkinter` and `ttk`. The main window creates a `ttk.Notebook` and instantiates one class per tab, each providing a `setup_gui()` method that declares widgets and binds callbacks. A consistent style theme is defined via `ttk.Style` (font, colours, progress bars, and buttons), yielding a uniform look-and-feel across the application. Figure 4.4.1 shows a representative screenshot of the interface for the acquisition processing. The first tab, labelled *Binary Plot* (`GraphApp`),

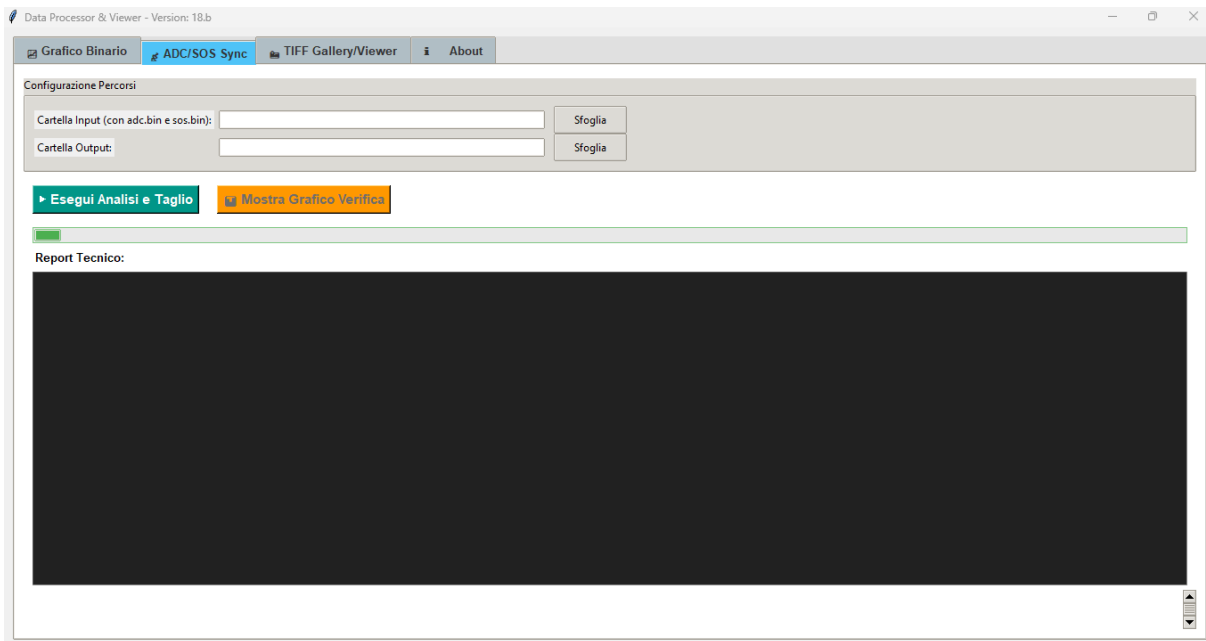


Figure 4.4.1: Screenshot of the GUI tab for the ADC/SOS Sync processing phase. The panel provides the dataset path selection, the execution controls, and a live technical log of the reconstruction steps.

is dedicated to low-level inspection of raw streams. It provides file browsing and an interactive plot window implemented by embedding Matplotlib (`TkAgg`) into a `tkinter Toplevel`. The plotting engine (`PlotEngine`) uses `np.memmap` to access the binary file on demand, allowing rapid navigation even for very long acquisitions. Interaction is provided by sliders for position and zoom, and by a crosshair readout that reports the sample index and amplitude under the cursor, enabling immediate sanity checks of SOS integrity, ADC saturation, and noise statistics, as it can be seen in the example in Figure 4.4.2.

The second tab, *ADC/SOS Sync* (*AdcSosSyncApp*), implements the full reconstruction

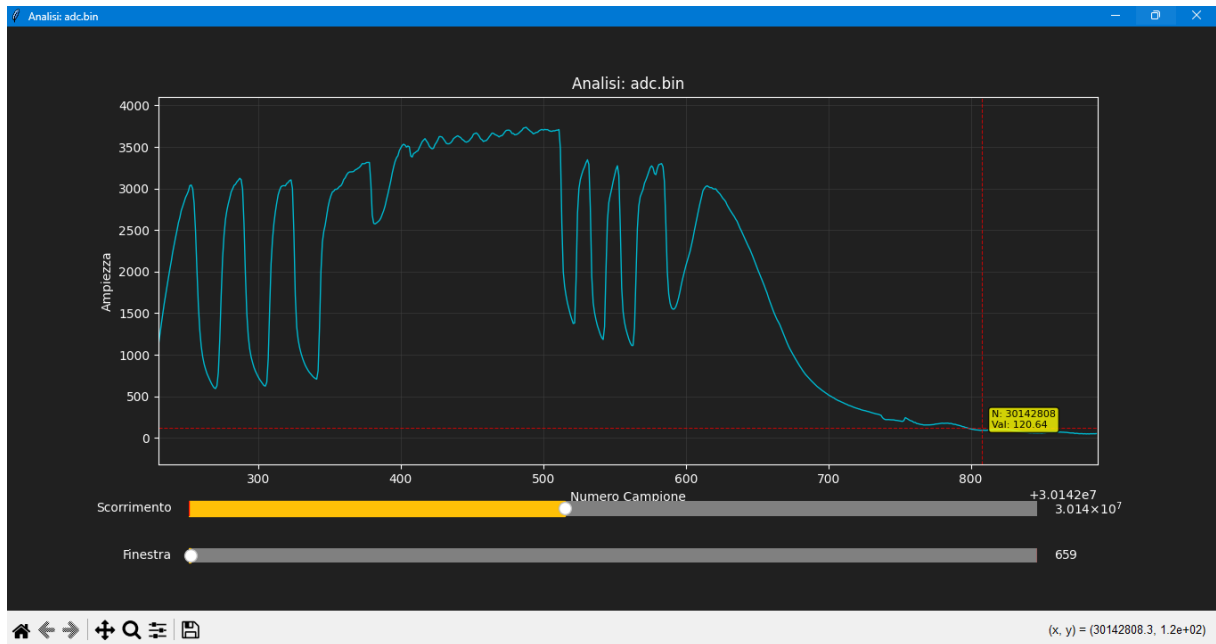


Figure 4.4.2: Screenshot of the GUI tab for the ADC/SOS graphs visualization.

pipeline and corresponds to the operational core of the software. The label “Analisi e Taglio” refers precisely to the two conceptual phases executed by `run_logic()`: first, the *analysis* of the synchronisation signal (*sos*) to obtain a reliable set of line boundaries (falling edges), identify a stable operating region, and align the SOS phase to the frame markers; second, the segmentation of the continuous ADC stream into line segments and frame intervals, followed by resampling, correction of corrupted lines, and writing of the final image files. The execution is launched by `start_analysis_thread()`, which spawns a daemon `threading.Thread` to avoid blocking the GUI event loop. Progress is communicated through a dedicated text console; the method `log()` schedules thread-safe UI updates via `master.after(0, ...)`, and an indeterminate `ttk.Progressbar` provides immediate feedback that processing is ongoing. A stop mechanism is implemented with a `threading.Event` (`stop_event`), which is periodically checked inside the frame loop to support user interruption without corrupting the UI state. The third tab, *TIFF Gallery/Viewer* (*GalleriaTIFF*), provides fast browsing and quantitative viewing of the reconstructed images. After selecting a folder, the program scans for `.tif/.tiff` files and displays the obtained images. In addition to the reconstruction pipeline, `Visualize.py` provides a dedicated cropping tool to isolate the effective illuminated field of view from the reconstructed frames. In the prototype, each image is reconstructed on a 1000×1000 grid. However, the outer regions can appear dark because, at large deflection angles and during polygon facet-to-facet transitions, the beam is partially clipped or temporarily

lost. Since these dark borders do not carry sample information, the software generates a cropped version of the frame intended for a better image visualization, while preserving the original uncropped image as the primary reconstruction output. The cropping region is estimated automatically using a robust, intensity-profile-based criterion that is insensitive to internal shadows within the illuminated spot. For a given frame $I(x, y)$, the algorithm computes a high-percentile envelope along each axis: for each column y , $p_y(y) = P_{95}(I(:, y))$, and for each row x , $p_x(x) = P_{95}(I(x, :))$, where P_{95} denotes the 95th percentile. The background and foreground levels of each profile are then estimated as $b = P_{10}(p)$ and $f = P_{90}(p)$, and a threshold is defined as $T = b + \alpha(f - b)$ (with $\alpha \simeq 0.2$). Columns (respectively rows) with $p_y(y) > T$ (respectively $p_x(x) > T$) are classified as belonging to the illuminated region. To avoid spurious detections, the algorithm retains the longest contiguous run of valid indices and expands it by a fixed pixel margin before clipping to the image bounds. The resulting bounding box $(x_0 : x_1, y_0 : y_1)$ is applied to generate the cropped image. In the GUI, the crop ROI is computed from a representative frame and then applied consistently to all frames of the same dataset. Cropped images can be exported in lossless 16-bit formats (TIFF/PNG), ensuring full spatial resolution. A screenshot of the Crop tab of the GUI is reported in Figure 4.4.3. This design choice proved essential to iteratively refine the reconstruction parameters and to mitigate recurrent artefacts such as isolated corrupted scan lines, while retaining a workflow compatible with the data formats produced by the acquisition hardware and the host-side recording scripts. The entire Python script of the visualization GUI is available in Appendix C.

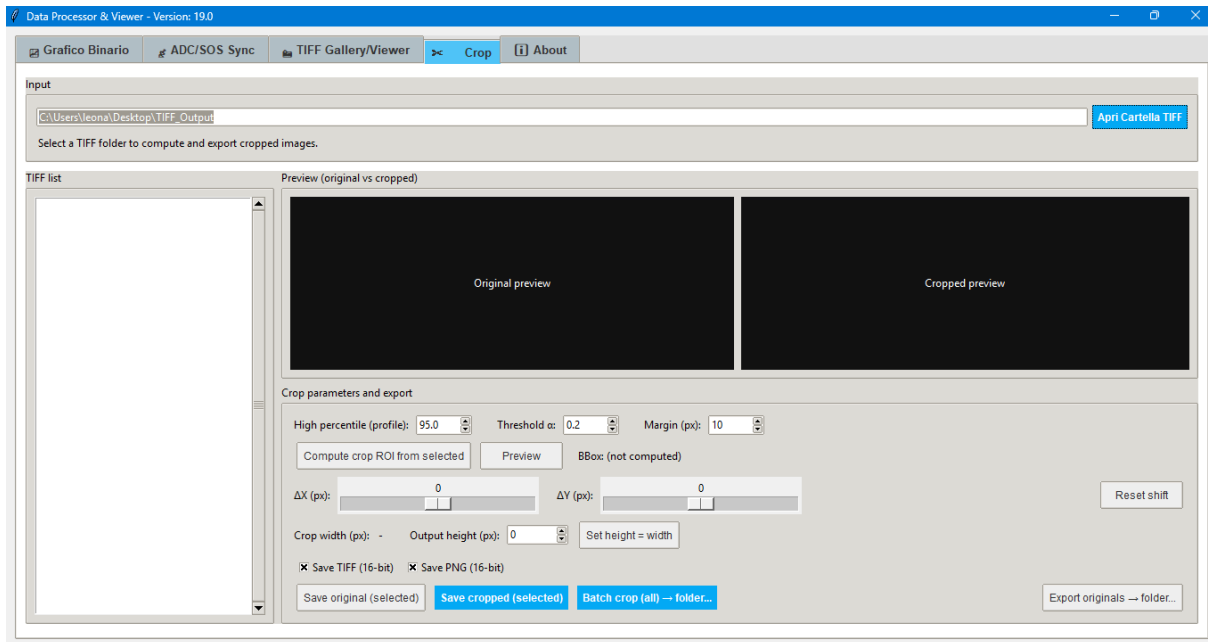


Figure 4.4.3: Screenshot of the GUI tab for image cropping.

5 | Assembly, Alignment and Performance Evaluation

5.1. Optical Alignment Procedure

This section describes in detail the optical alignment procedure and the mechanical assembly of the individual components, using either commercial opto-mechanical mounts or custom-designed, 3D-printed supports. The setup is built on a Standa optical table with a 25 mm hole grid and M6 threaded holes for mounting optical components, ensuring rigidity and reduced vibrations. Unless otherwise specified, all optical elements are mounted on the table using standard Thorlabs hardware: lens mounts for lenses, posts with M4 threading, post holders, baseplates, and clamps fixed to the optical table using M6 screws. The first element mounted in the position shown in the setup scheme in Figure 3.3.2, is the CNI Lasers laser source, shown in Figure 5.1.1. It is fixed on four posts so that the



Figure 5.1.1: MDL-III-980L laser source and PSU-III-LED controller by CNI Lasers.

outgoing beam is aligned with a row of holes on the optical table for convenience. In addition, using a small bubble level and simple tests performed by observing the beam position on the Thorlabs webcam DCC1545M 2D sensor at different distances from the source, the output beam is verified to be parallel to the optical table, without upward or downward tilt. As mentioned in Section 3.2, the beam proved to have a very elliptical shape. A screenshot of the beam shape acquired by the webcam in the proximity of the source is shown in Figure 5.1.2. The first phase of the optical alignment consists of integrating the scanners and aligning the two relay systems in a $4-f$ configuration, while the epi-detection section is integrated only at a later stage. Therefore, after the laser, the first element encountered by the beam is the first scanner: the rotating polygon mirror from Precision Laser Scanning. As shown in Figure 2.5.2, the polygon mirror and its motor are mounted together with their PCB containing the required electronic interfacing. It is therefore necessary to fabricate a support that can rigidly hold the mirror while allowing adjustment of its spatial orientation, to ensure that the scan performed by the mirror is



Figure 5.1.2: Elliptical shape of the beam acquired by the DCC1545M webcam.

effectively horizontal (i.e., parallel to the optical table plane). The solution is shown in Figure 5.1.3. After verifying that the rotating polygon mirror is tilted correctly such that

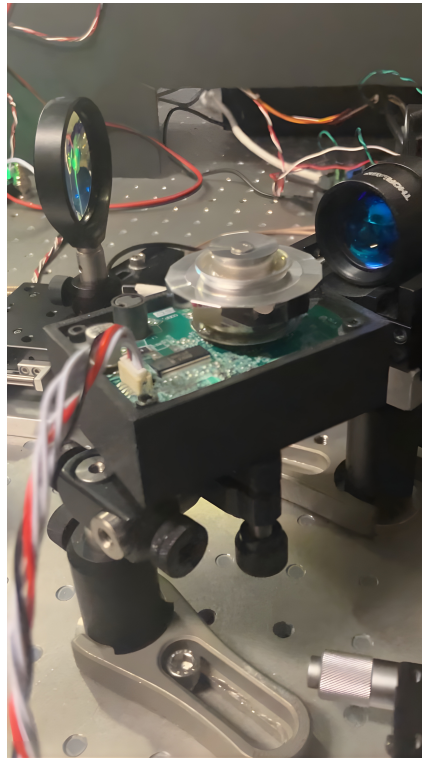


Figure 5.1.3: Polygon Mirror custom-designed 3D-printed mount.

the reflected beam remains at the same height above the optical table, the first $4-f$ relay system must be aligned. The lenses forming this $4-f$ system must be precisely centered with respect to the scan range, with the first lens at a distance f from the reflective facet of the rotating mirror and the second one separated by a distance equal to $2f$. If the lens

positions are correct, at a distance f from the second lens there should be a “pivot” point crossed by beam during scanning at each scan angle of the entire range, as represented in Figure 2.1.3. To identify the center of the horizontal scan region, the most robust criterion is symmetry: the center is reached when the positive and negative angular deflections produced around the nominal facet normal are mapped to equal excursions on a calibrated grid test target. Practically, record the leftmost and rightmost positions of the scanned line and adjust the polygon’s angular “zero” by small yaw rotations of the polygon mount (rotation around the vertical axis, which changes the average facet normal seen by the incoming beam). The correct centering corresponds to equal margins to the left and right before clipping/vignetting, and to a scan that reaches the same effective field of view on both sides. Then, an auxiliary lens with the same focal length of (30 mm) as the lens to be placed at distance f from the polygonal mirror is used. By placing this auxiliary lens on the beam path before the mirror (which is not rotating during this procedure), the light is focused precisely onto the mirror surface. Then placing the lens of focal length f after the polygonal mirror, the beam focused on the mirror and reflected from its surface will exit the second lens collimated if and only if that lens is positioned exactly at distance f (30 mm) from the mirror, as in the configuration shown in Figure 5.1.4. After removing the auxiliary lens, to complete the relay, the second 30 mm lens must

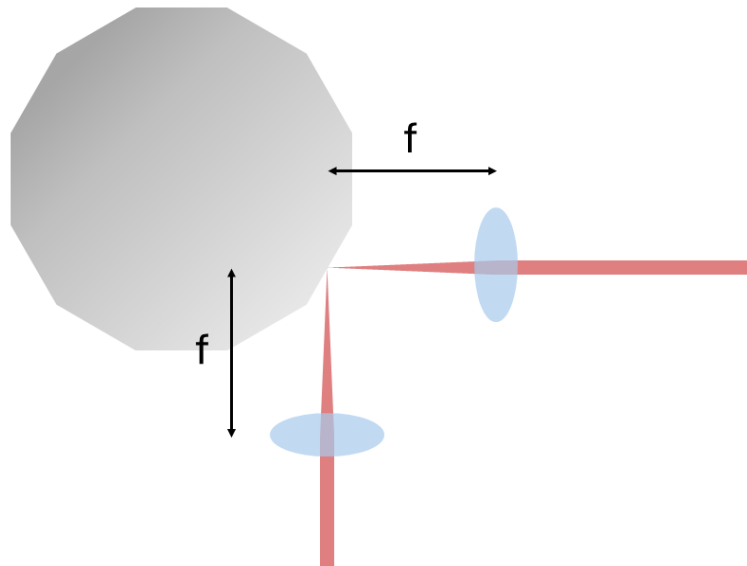


Figure 5.1.4: Alignment procedure to place the first lens of the 4-f system to relay the horizontal scan on the galvanometric mirror.

be positioned exactly at distance $2f$ (60 mm) from the first one. The first lens focuses

the collimated beam reflected by the rotating mirror at distance f . To ensure that the second lens is positioned at exactly $2f$ from the first, it is sufficient to finely adjust its position with a translation stage until the output beam is collimated, since this condition occurs only when the lens separation equals $f + f = 2f$, as shown in Figure 5.1.5. As

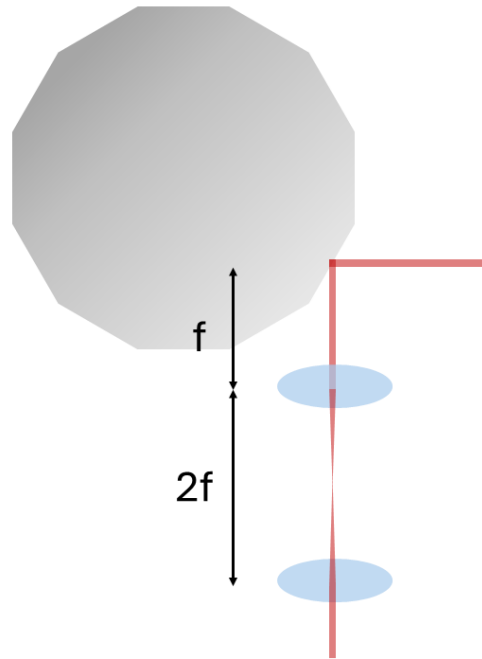


Figure 5.1.5: Alignment procedure to complete with the second lens the 4- f system to relay the horizontal scan on the galvanometric mirror.

mentioned above, a “pivot” point exists at a distance f from the second lens, crossed by each scanned beam exiting the 4- f system when the polygonal mirror motor is switched on and actively scanning. This point can be located coarsely using an alignment card or more precisely using a webcam with a 2D sensor, observing that the laser spot appears still at that location. The galvanometric mirror is placed precisely at this pivot point, taking particular care to center the beam on the middle of its reflective surface. In this phase, it is essential that the galvo reflective surface lies exactly at the pivot point: if it is placed slightly closer or farther, the reflection on the galvo will not correspond to a stationary point, but rather to a line, implying that some rays will not be centered on the galvo and will contribute to optical non-idealities. The galvanometric mirror oscillates vertically, describing a vertical scan line which, combined with the horizontal scan performed by the rotating mirror, completes the 2D raster scanning that must be relayed to the objective back aperture through a second 4- f lens system. Unlike the first relay, this second 4- f system must also introduce lateral magnification of the laser beam to achieve proper overfilling of the objective back aperture as calculated in Section 3.2. Therefore, it

uses two lenses with different focal lengths f_1 and f_2 . Using the same procedure described for the first $4-f$ system, the lens with $f_1 = 30$ mm is positioned first, followed by the lens with $f_2 = 150$ mm at a separation of

$$f_1 + f_2 = 180 \text{ mm.}$$

Also in this case, using translation stages to finely adjust the lenses positions helps a lot in the alignment procedure. At the output of this second $4-f$ relay, the beams are collimated and scanned both horizontally and vertically. As for the first $4-f$ system, all scan rays pass through a pivot point: each ray corresponds to a specific angle in both horizontal and vertical directions. This point, located at a distance f_2 from the second lens, can be identified coarsely using an alignment card or more precisely with a webcam, since the laser spot appears stationary at that location despite scanner motion. A photo of the two relay $4-f$ systems aligned with the scanners is reported in Figure 5.1.6. The

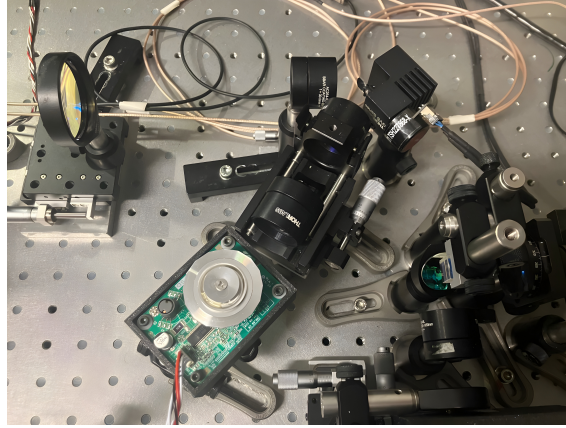


Figure 5.1.6: Photo of the two scanners together with the aligned $4-f$ relay systems.

objective back aperture must be located at this pivot point. In this configuration, angular deflections of the beam incident at the back aperture are converted into the corresponding horizontal and vertical displacement of the laser spot at the sample plane, as already explained in Figure 2.1.6. The objective is installed in a custom-designed, 3D-printed case with the quarter-wave plate fixed immediately before the back aperture and mounted on top of the motorized Zaber Z translation stage, as shown in Figure 5.1.7. To keep the experimental campaign consistent with the primary objectives of this thesis, the optical alignment and validation activities were intentionally focused on the illumination and scanning chain up to the pupil-conjugate plane of the second $4f$ relay. In particular, the work targeted (i) the mechanical integration and opto-mechanical alignment of the polyg-

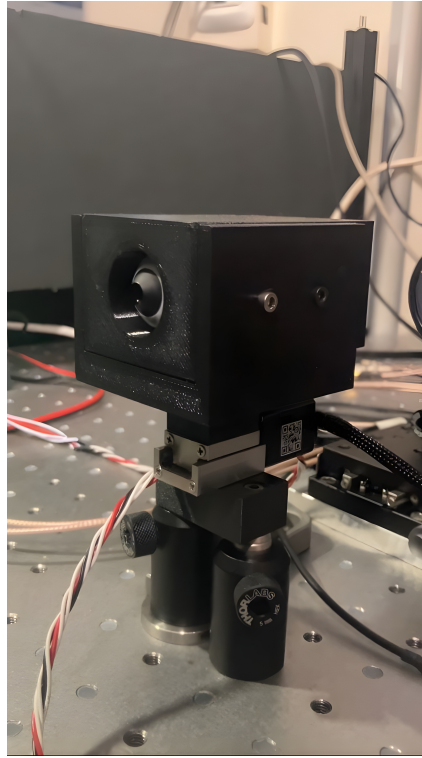


Figure 5.1.7: Photo of the objective installed into a 3D-printed custom-designed case on top of the Zaber motorized translation stage. Behind the objective (not visible in the photo) the QWP is attached directly to the back aperture.

onal scanner and galvanometric mirror, (ii) the alignment of the two $4f$ relays required to preserve the scanner pivot conjugation and to deliver a collimated, two-axis scanned beam at the objective back-aperture plane, and (iii) the end-to-end verification of scanner synchrony through the electronic interfacing, control software, and the image reconstruction algorithm implemented on the host computer. By first demonstrating stable raster scanning and correct pupil relay in a transmission-like configuration, the alignment can be quantified and debugged with minimal ambiguity before introducing the additional degrees of freedom associated with epi-detection. Accordingly, the measurements reported in Section 5.2 and in Chapter 6 were performed in transmission, without the objective lens in place. The sample was positioned at the focal plane of the second $4f$ system, while the transmitted light was collected by a large-area (single-pixel) detector placed at the downstream pivot (pupil-conjugate) plane. This geometry preserves the essential timing and segmentation conditions required for image formation—namely, a well-defined mapping between scanner angles and sampled detector signal—and therefore provides a rigorous experimental validation of the scanning optics, synchronization electronics, and reconstruction software under controlled conditions. The epi-detection arm (polarizing

beam splitter, focusing lens, confocal pinhole and detector) was nonetheless mechanically integrated, and a preliminary optical consistency check was performed to verify correct beam routing. Specifically, by placing a mirror at the objective focus position, the back-reflected beam was observed to retrace the illumination path and to be redirected by the polarizing beam splitter into the detection branch, confirming that the separation of illumination and return paths was correctly implemented. A full confocal alignment of the detection arm (pinhole centering, detector conjugation, and fine optimization of return-path aberrations) is therefore a well-defined next step building directly on the validated illumination-and-scanning baseline established in this work. At this point, the detection arm is outlined. First, a polarizer is inserted immediately after the laser source to improve as much as possible the polarization purity of the light entering the PBS. Next, the PBS is mounted on a sample-holder fixed to a post and aligned with the outgoing beam so that vertically polarized light (the polarization of the laser output) is transmitted and horizontally polarized light coming from the sample is reflected downwards towards a 45 degree mirror that reflects it again into the detection arm, while the horizontally polarized light originating from the sample (even if it is just a small fraction of the optical power) is reflected upwards the PBS mount. The light reflected by the PBS is a collimated beam that is focused by a lens with $f = 35$ mm onto a $100\text{ }\mu\text{m}$ -diameter pinhole. Immediately behind the pinhole, the APD detector is placed. It is also housed in a 3D-printed case that keeps it close to its high-voltage supply, facilitating electrical connections and improving overall compactness. A photo of the detection arm is reported in Figure 5.1.8 Finally,

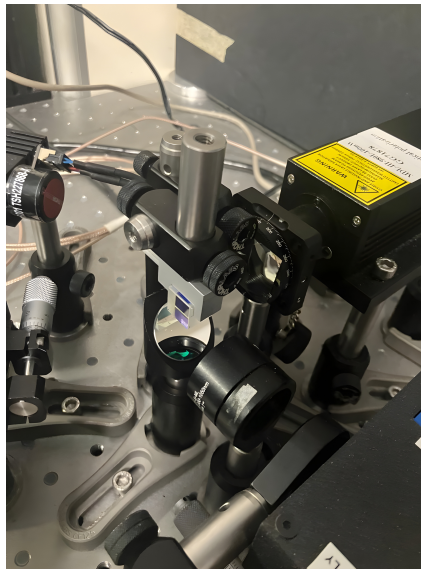


Figure 5.1.8: Photo of the detection arm constituted by PBS, 45 degree mirror, focusing lens, pinhole and detector. The polarizer placed outside the laser source is also visible.

the SOS optical system is aligned, for simplicity, on a different facet of the rotating mirror than the one used by the main beam, in order to facilitate alignment given the high density of optical elements near the polygon mirror. Both the SOS laser diode and the SOS detector are installed on custom-designed, 3D-printed supports that are then fixed on posts. A photo of the SOS system is reported in Figure 5.1.9. All electronic elements—

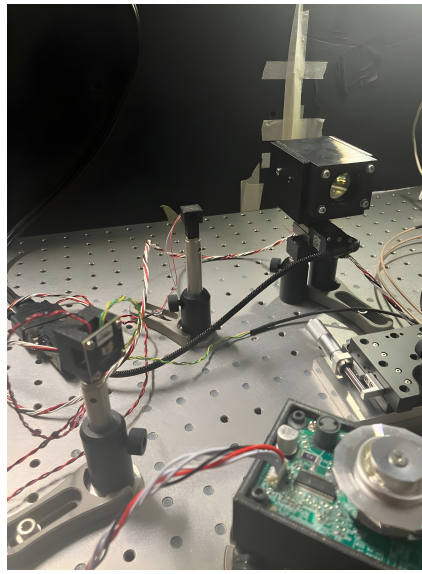


Figure 5.1.9: Photo of the SOS system: closer on the left, the SOS detector on its custom-designed mount; far in the back, the SOS laser, mounted in its 3D-printed support.

such as the STEMLab 125-14, the auxiliary board used to amplify the STEMLab outputs, and the Zaber stage controller—are placed and fixed onto the optical table according to space constraints and practical convenience. The photo of the complete setup built on the optical table is reported in Figure 5.1.10.

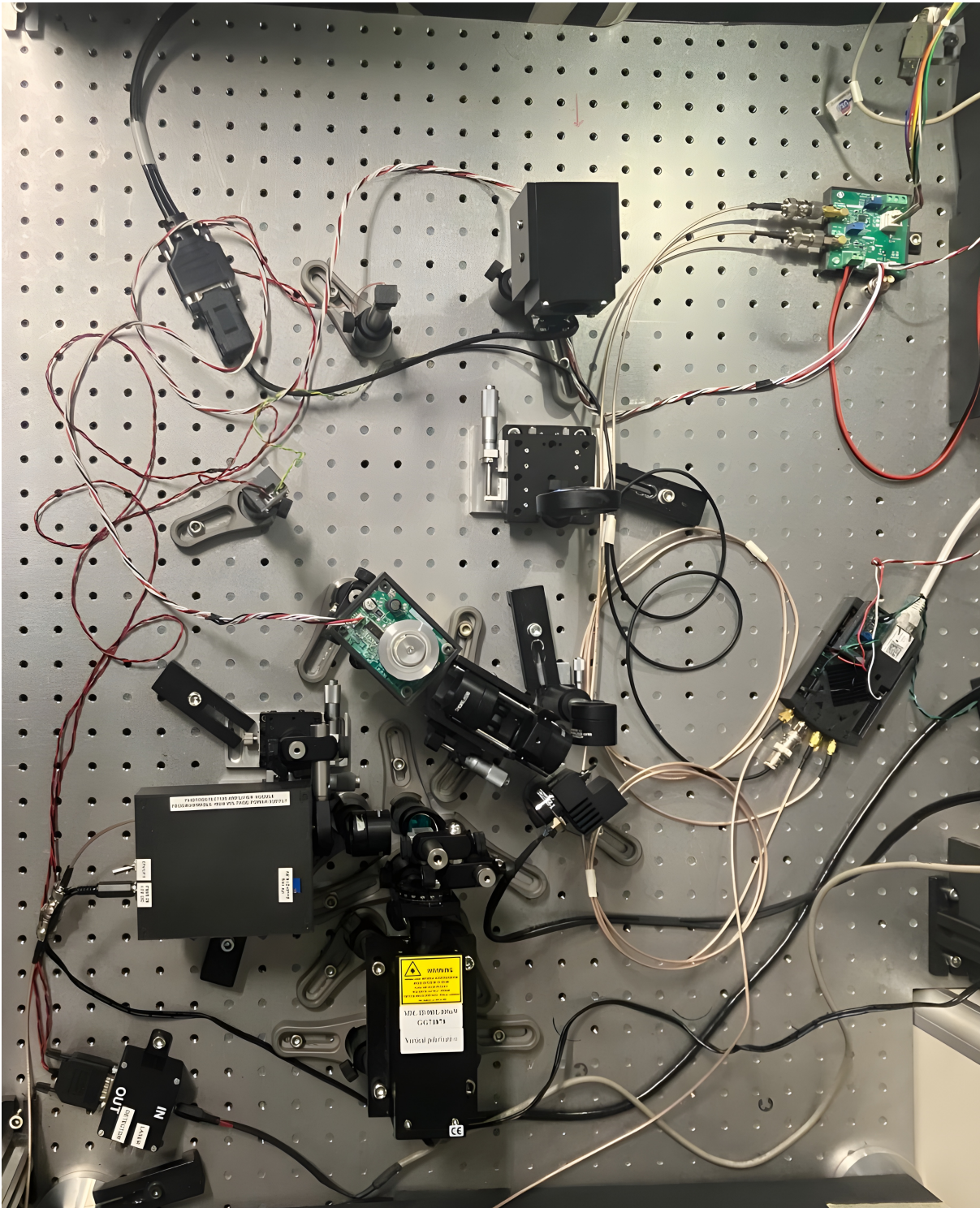


Figure 5.1.10: Photo of the entire setup with all the optical elements mounted on the table. On the right, the STEMLab 125-14 (bottom) and the Amplifier Board (top) are shown.

5.2. Tests on the Scanning Raster Pattern Synchronization

The experimental activity reported in this section was conceived as a staged validation of the prototype, focusing on the illumination and scanning chain up to the objective back-aperture plane (i.e., the back focal plane of the second $4f$ relay), including the assessment of the functioning of the electronic configuration of the scanners, their mutual alignment, the alignment of the two $4f$ relays that preserve the pivot conjugation, the control software, and the host-side image reconstruction algorithm. Within this scope, the detection arm was mechanically integrated and optically verified in terms of beam routing, but it was not fully aligned as a confocal reflection channel. The measurements discussed here were therefore performed in transmission in order to directly validate the raster scan synchronization and image reconstruction under controlled conditions, in the configuration shown in Figure 5.2.1. The transmitted signal was acquired with a Thor-

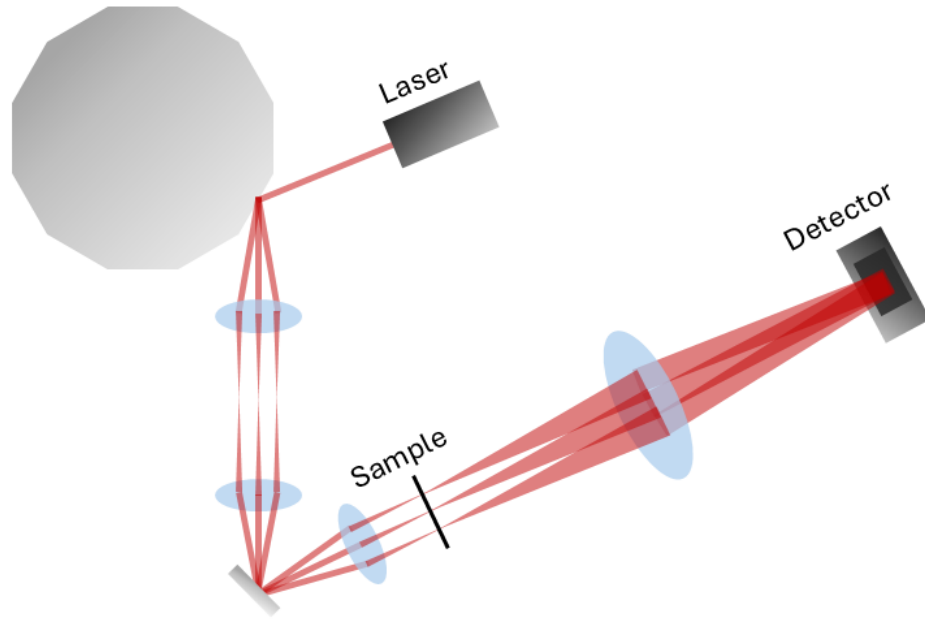


Figure 5.2.1: Configuration of the experimental tests detailed in this Section.

labs PDA36A2 amplified Si photodiode (datasheet in Appendix A.14), placed at the pivot (pupil-conjugate) plane at the output of the second $4f$ relay and mounted on a translation stage to finely adjust its axial position with respect to the second relay lens. Its output is connected to the STEMLab fast analog input channel CH1 via BNC cable. For these tests the detector gain was set to its minimum value (0 dB), so as to exploit the maximum available bandwidth (12 MHz) and preserve the temporal fidelity of the wave-

form within each scan line. Since the STEMLab analog input presents a high impedance ($1\text{ M}\Omega$) while the detector output is specified for a $50\text{ }\Omega$ environment, a $50\text{ }\Omega$ terminator is placed in parallel at the acquisition input in order to provide the correct load and to reduce the effective RC time constant associated with the cable parasitic capacitance, thereby improving the high-frequency response of the acquisition chain. As a first sanity check, the scanners were operated with no sample in the beam path, so that the photodiode measured only the scanned beam footprint. The reconstructed full-frame image (Fig. 5.2.2) exhibits a bright scan envelope surrounded by dark borders, which are expected because at large deflection angles and during polygon facet-to-facet transitions the beam is partially clipped or temporarily lost, yielding time intervals with no detected power. This motivates the systematic use of the cropping tool described in Section 4.4:

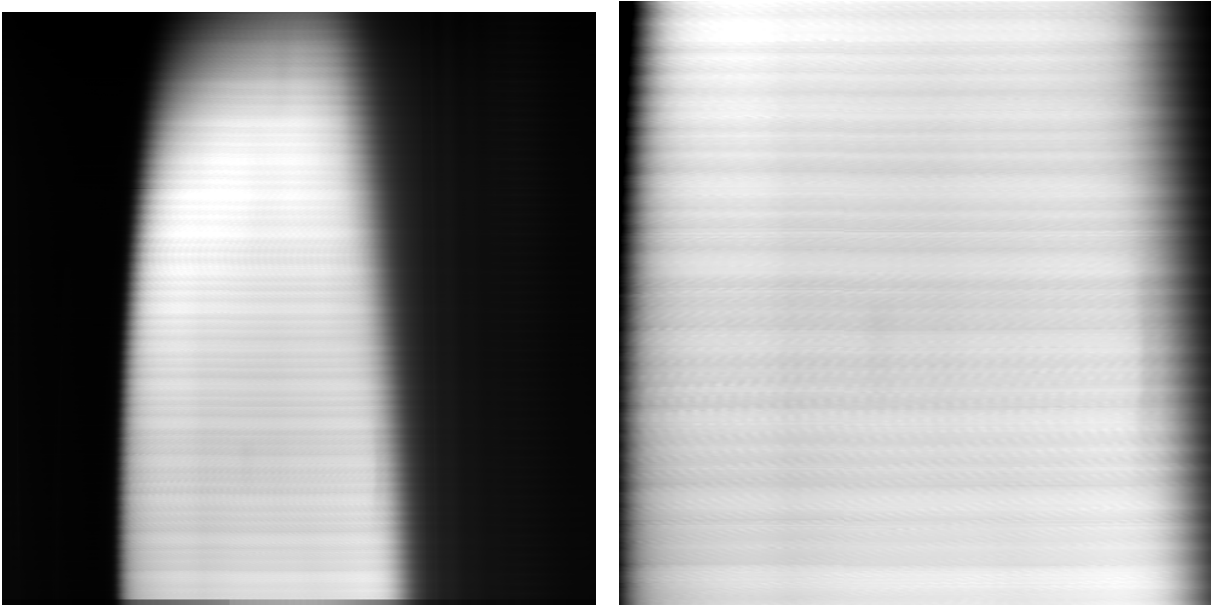


Figure 5.2.2: Empty-field test (no sample): reconstructed frame showing the illuminated scan envelope and dark borders due to clipping/facet transitions (left), and the corresponding cropped view isolating the effective field of view used for visualization and figure preparation (right).

the cropped frame in Fig. 5.2.2 isolates the effective illuminated region and suppresses the non-informative dark margins, improving interpretability without altering the underlying reconstruction. In this transmission configuration, the reconstructed scan envelope appears only partially filled because the single-pixel detector samples a limited portion of the scanned beam footprint, set by the detector active area and its alignment at the pupil-conjugate plane. The resulting field therefore corresponds to the intersection between the illuminated scan envelope and the detector collection region. The corresponding oscilloscope trace acquired on a Teledyne LeCroy HDO6104 (datasheet in Appendix A.15) is

shown in Fig. 5.2.3: the waveform displays a high-level plateau when the scanned beam overlaps the detector active area and a low-level interval when the beam is outside the collection region. From the timebase ($20 \mu\text{s}/\text{div}$) the repetition period between successive

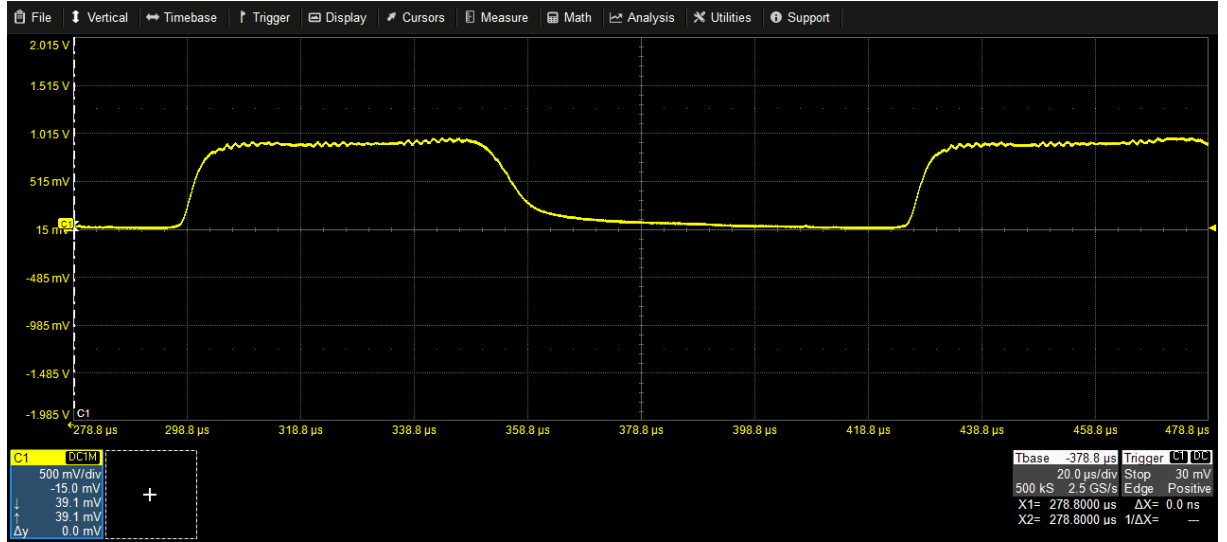


Figure 5.2.3: Oscilloscope trace (Teledyne LeCroy HDO6104) of the photodiode signal in the empty-field test. The plateau corresponds to the useful portion of a scan line in which the beam overlaps the detector active area, while the low-level interval corresponds to non-illuminated time due to scan geometry and polygon facet transitions.

plateaus is on the order of $\sim 1.3 \times 10^2 \mu\text{s}$, while the approximately flat “useful” portion within a plateau spans $\sim (5-6) \times 10^1 \mu\text{s}$, indicating a duty cycle of ~ 0.4 for the effective angular sweep contributing to image formation. This observation is consistent with the full-frame reconstruction and provides an independent time-domain validation of the line segmentation strategy: the reconstruction maps a fixed number of horizontal pixels onto the time interval in which the beam is effectively present on the detector, while discarding (or equivalently mapping to the dark borders) the non-illuminated portions. After validating the empty-field behaviour, a simple high-contrast object (an Allen key) was positioned in the focal plane of the second $4f$ relay, so that the transmitted power was modulated primarily along one direction. The reconstructed image in Fig. 5.2.4 shows a well-defined dark stripe corresponding to the object shadow, with straight edges and continuity across scan lines, demonstrating that the polygon/galvo timing, the STEM-lab sampling and the frame/line segmentation are sufficiently stable and synchronized to reproduce a quasi-1D feature without line dropouts or inter-line shear large enough to disrupt the object boundary. More complex tests were then performed with a USAF 1951 glass slide resolution target (Edmund Optics; datasheet in Appendix A.17), which is a standardized pattern of three-bar elements arranged in groups of increasing spatial

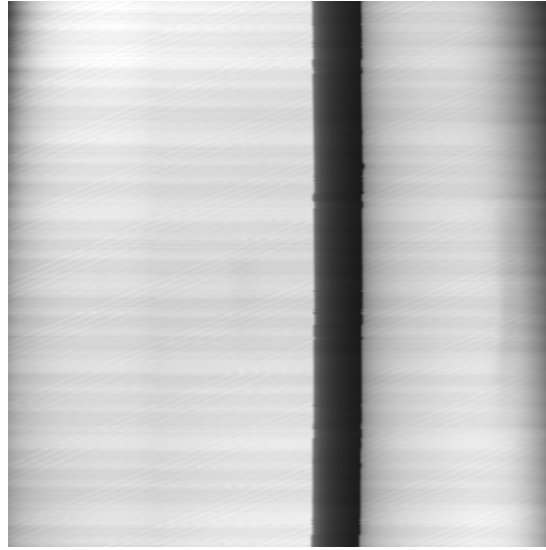


Figure 5.2.4: Transmission test on a simple high-contrast object (Allen key) placed at the focal plane of the second $4f$ relay. The straight, continuous shadow boundary provides a direct qualitative validation of raster stability and correct line-to-line synchronization.

frequency and widely used to assess the resolving capability and distortion of imaging systems. A first qualitative assessment aimed at reproducing the “Edmund” lettering on the target. Because of the limited instantaneous field collected in the transmission configuration, two adjacent reconstructions were required to cover the entire word (Fig. 5.2.5), and the resulting frames confirm correct raster generation over extended scan excursions, while also evidencing a gradual degradation toward the edge of the illuminated envelope consistent with increased aberrations and reduced effective collection at larger scan angles. Finally, the bar patterns of the USAF target were imaged to assess the highest spatial frequency transmitted through the scanning and reconstruction chain (Fig. 5.2.6). In the corresponding oscilloscope trace (Fig. 5.2.7) the bar triplets manifest as repeated intensity dips/peaks within the useful portion of the line, providing a direct time-domain counterpart of the spatial modulation observed in the reconstructed image and confirming that the detected signal bandwidth is sufficient to encode the target contrast within a single scan line.

Although these measurements are not confocal reflection images and do not include the objective, it is still meaningful to discuss a lateral resolution *in the validation plane* (the focal plane of the second $4f$ relay) as the smallest resolvable bar pattern on the USAF target, because this quantifies the effective spot size and the ability of the timing/reconstruction pipeline to preserve spatial modulation. In the USAF 1951 standard, each bar pattern is

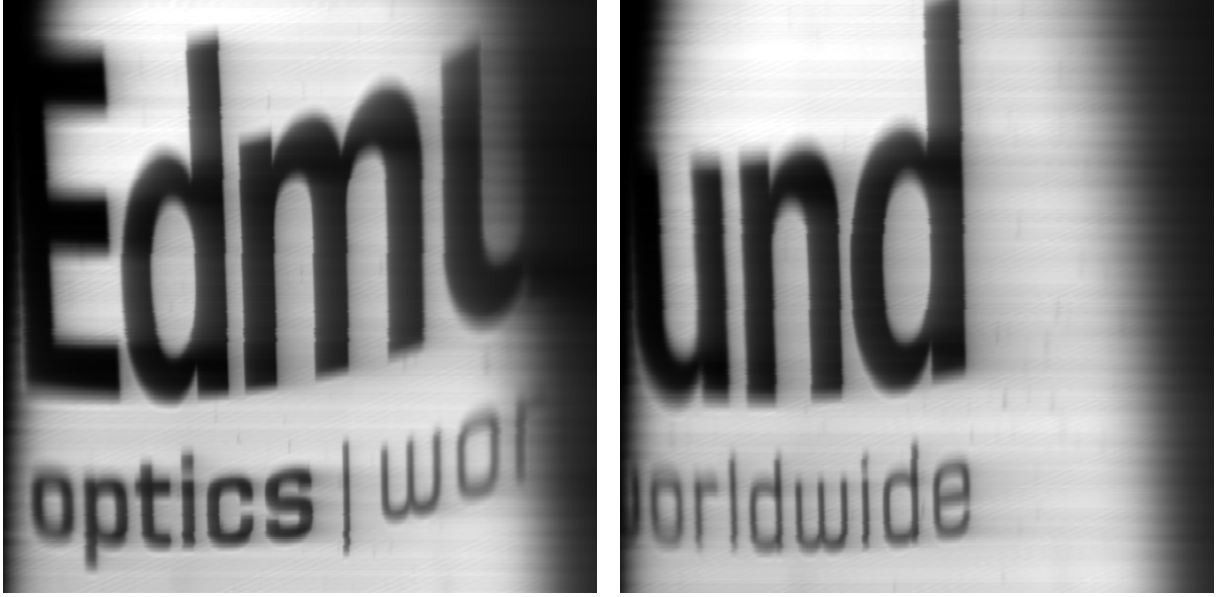


Figure 5.2.5: USAF target lettering test (two adjacent frames): reconstruction of the “Edm” (left) and “und” (right) portions of the “Edmund” writing. The result validates large-area raster consistency in the transmission configuration and highlights increasing distortions toward the edge of the illuminated envelope.

identified by a group index g and an element index $e \in \{1, \dots, 6\}$, and the corresponding spatial frequency in line pairs per millimeter (lp/mm) is

$$f_{lp}(g, e) = 2^{g+(e-1)/6} \text{ lp/mm}. \quad (5.1)$$

A line pair corresponds to one dark bar plus one bright spacing. Therefore, the line-pair period and the single-bar width are, respectively,

$$p = \frac{1}{f_{lp}}, \quad w = \frac{p}{2} = \frac{1}{2f_{lp}}. \quad (5.2)$$

From the reconstructed image in Fig. 5.2.6, the finest pattern that remains clearly separable can be stated conservatively as Group 3, Element 6, while an optimistic interpretation suggests visibility up to Group 4, Element 2–3. Using Eqs. (5.1)–(5.2), these correspond to

$$(g, e) = (3, 6) : f_{lp} = 14.25 \text{ lp/mm}, \quad p = 70.15 \text{ } \mu\text{m}, \quad w = 35.08 \text{ } \mu\text{m},$$

$$(g, e) = (4, 2) : f_{lp} = 17.96 \text{ lp/mm}, \quad p = 55.68 \text{ } \mu\text{m}, \quad w = 27.84 \text{ } \mu\text{m},$$

$$(g, e) = (4, 3) : f_{lp} = 20.16 \text{ lp/mm}, \quad p = 49.61 \text{ } \mu\text{m}, \quad w = 24.80 \text{ } \mu\text{m}.$$

Accordingly, the transmission validation performed in the focal plane of the second $4f$

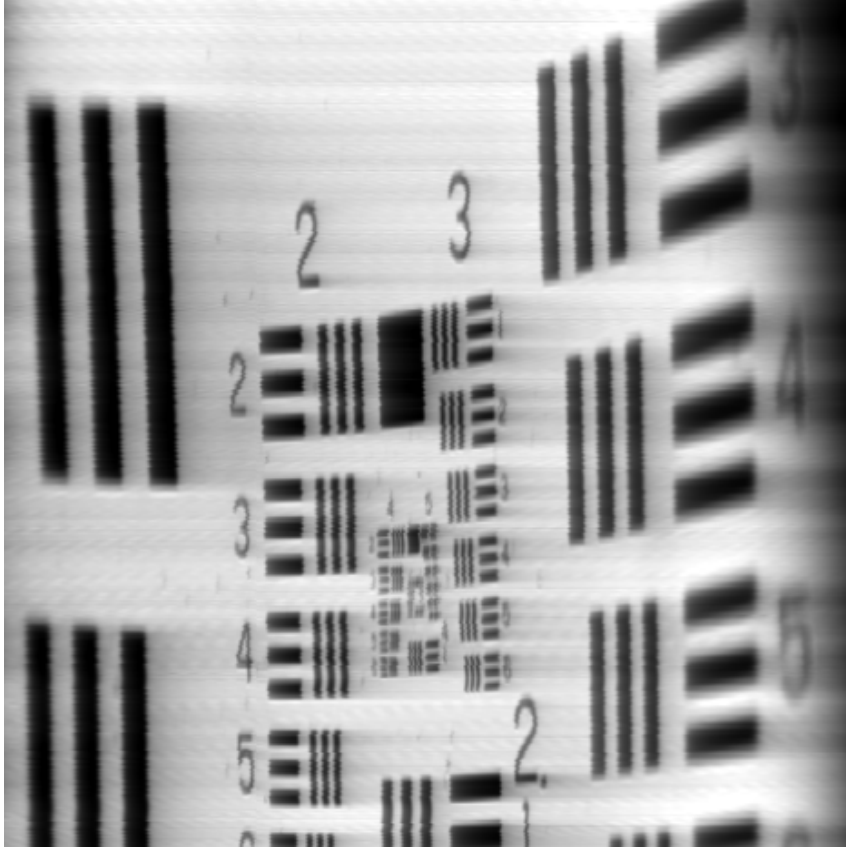


Figure 5.2.6: USAF 1951 resolution target bar-pattern test. The bar triplets produce characteristic intensity modulations within the useful portion of each scan line, confirming bandwidth adequacy and correct timing/reconstruction of high-spatial-frequency features in the test plane.

relay indicates a test-plane lateral resolving capability on the order of

$$\delta_{\perp, \text{test}} \approx (25\text{--}35) \mu\text{m}, \quad (5.3)$$

where the interval reflects the conservative versus optimistic readout of the smallest resolvable USAF element. It is important to stress that this value should not be interpreted as the final microscope resolution, because the present configuration does not include the objective and is not operated as an epi-confocal system: the measured contrast is affected by the effective numerical aperture of the second $4f$ focusing lens, by the finite collection geometry of the single-pixel detector placed at a pupil-conjugate plane, and by scan-angle-dependent clipping at the edges of the illuminated envelope. Nevertheless, Eq. (5.3) is still a meaningful quantitative figure-of-merit for this thesis, because it verifies that the scanner synchronization, acquisition bandwidth, and reconstruction pipeline preserve spatial modulation well into the tens-of-micrometres scale at the chosen scan

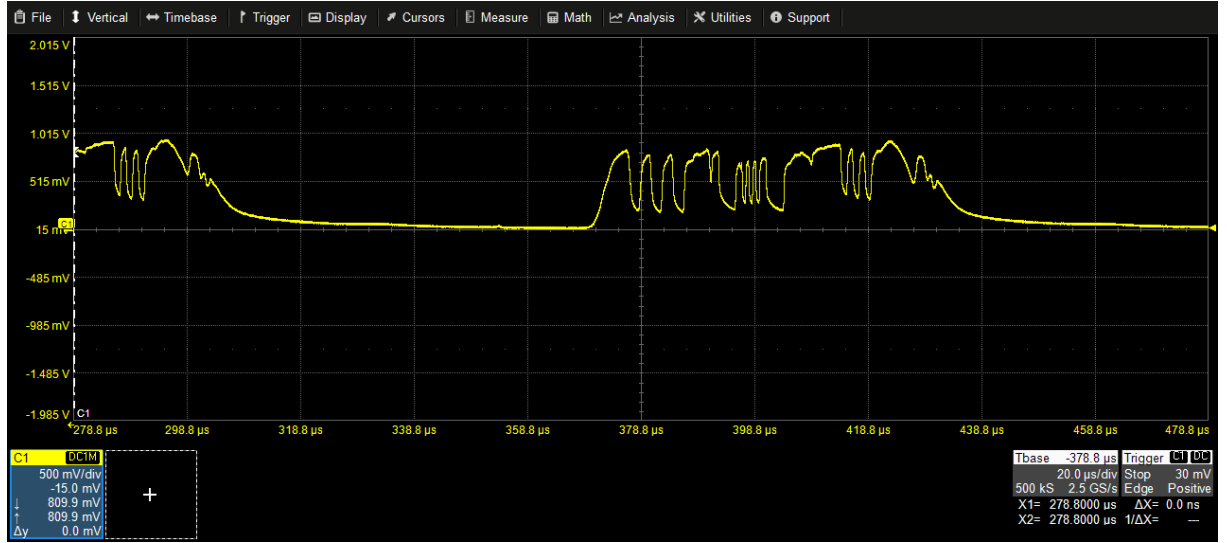


Figure 5.2.7: Oscilloscope trace of the transmitted photodiode signal during the USAF 1951 test. The modulations correspond to the bar triplets, confirming bandwidth adequacy and correct timing/reconstruction of high-spatial-frequency features in the test plane.

speed.

For comparison, once the objective ($\text{NA} = 0.8$) is introduced and the confocal detection arm is aligned, the diffraction-limited lateral resolution at $\lambda = 980 \text{ nm}$ is expected to approach the Rayleigh criterion

$$\delta_{\perp, \text{obj}} \simeq 0.4 \frac{\lambda}{\text{NA}} \approx 0.4 \frac{980 \text{ nm}}{0.8} \approx 0.49 \text{ } \mu\text{m}. \quad (5.4)$$

Therefore, while the USAF measurements reported here primarily validate scanner synchrony, waveform bandwidth, and reconstruction correctness (demonstrating that the raster preserves spatial modulation up to the target element (3,6) in the test plane), the completed reflectance confocal configuration is expected to achieve sub-micrometer lateral resolution consistent with the objective NA and the design wavelength, with the present results providing the necessary end-to-end timing and reconstruction baseline on which the confocal detection arm can be aligned and optimized.

6 | Experimental Results and Discussion

6.1. Biological Samples Imaging Tests

The final experimental validation of the prototype was carried out on biological specimens in the same transmission configuration described in Section 5.2 and in Figure 5.2.1, namely with the rotating polygon mirror and galvanometric scanner generating the raster pattern, the two aligned $4f$ relay systems preserving the pivot conjugation, and the single-pixel Thorlabs PDA36A2 photodiode positioned at the pupil-conjugate plane of the second relay. No objective was inserted in the optical path, and the samples were placed in the focal plane of the second $4f$ system, so that the reconstructed images represent the spatial modulation of the transmitted intensity across the scanned beam footprint. Two stained mouse limb (hip joint) sections mounted on glass slides were investigated, labelled *AI-46 RGB* and *AI-53 CL RGB*. Photographs of the glass slides are reported in Figs. 6.1.1a and 6.1.1b, respectively. The macroscopic images show thin histological sections stained in pink/red tones, typical of standard histological preparations, with the joint region and surrounding connective tissue clearly visible at the centimeter scale.



(a) Photograph of the stained mouse limb section labelled AI-46 RGB mounted on a glass slide.



(b) Photograph of the stained mouse limb section labelled AI-53 CL RGB mounted on a glass slide.

Figure 6.1.1: Photographs of the stained mouse limb sections mounted on glass slides.

The reconstructed image of sample AI-46 is shown in Fig. 6.1.2. Within the illuminated

region the transmitted intensity exhibits structured variations that follow the underlying biological morphology. Elongated features corresponding to bone and joint structures can be identified, together with finer internal textures likely associated with trabecular bone patterns and surrounding soft tissue. The contrast is moderate but clearly above the noise floor, indicating that the bandwidth of the acquisition chain and the temporal sampling strategy are sufficient to preserve spatial modulation at the tens-of-micrometres scale.

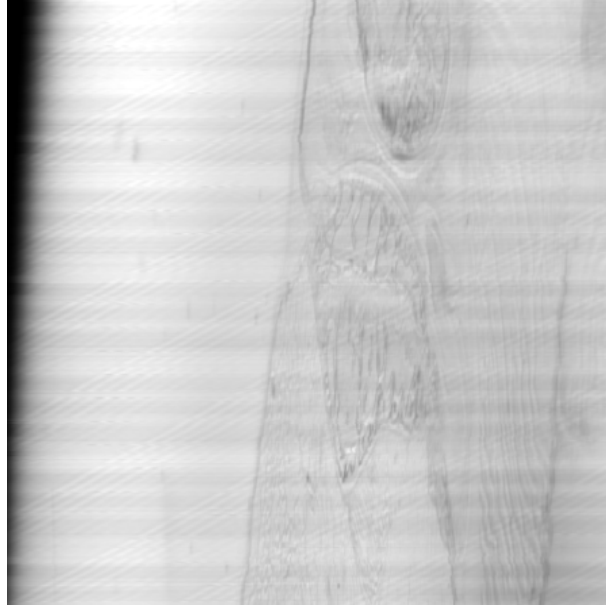


Figure 6.1.2: Transmission reconstruction of sample AI-46. Cropped image isolating the effective field of view used for analysis and visualization.

The second specimen, AI-53 CL RGB, is reported in Fig. 6.1.3 as a cropped reconstruction. Also in this case, distinct anatomical regions are recognizable through variations in transmitted intensity, including curved interfaces and internal microstructural patterns. Compared to the simpler test objects of Section 5.2, the biological tissue introduces spatially varying absorption and scattering, resulting in a more complex grayscale texture. Nevertheless, the continuity of structural features across successive scan lines confirms the correct synchronization between the polygonal mirror and the galvanometric scanner and the absence of significant line-to-line timing jitter in the reconstructed frame. The observable microstructures appear consistent with the previously estimated test-plane lateral resolving capability on the order of a few tens of micrometres, as derived from the USAF target measurements. While the present configuration does not yet exploit the high-numerical-aperture objective and the confocal pinhole foreseen for the final reflectance implementation, the experiments reported here provide an intermediate milestone: they validate the end-to-end temporal-to-spatial mapping (scanner drive $\rightarrow$ detector waveform $\rightarrow$ frame markers $\rightarrow$ reconstructed raster) on real, highly heterogeneous biological tis-

sue. In the completed epi-confocal configuration, the same scanning and reconstruction backbone will operate unchanged, whereas the addition of the objective, the polarization-based separation of illumination and return paths, and the confocal spatial filtering are expected to (i) compress the illumination spot to the diffraction-limited scale, (ii) suppress out-of-focus background and multiply scattered light, and (iii) enhance edge contrast and fine morphological detail. Therefore, these transmission-mode results can be interpreted as a system-level verification of the most timing-critical and software-intensive components, effectively de-risking the subsequent alignment and optimization of the reflection detection arm.

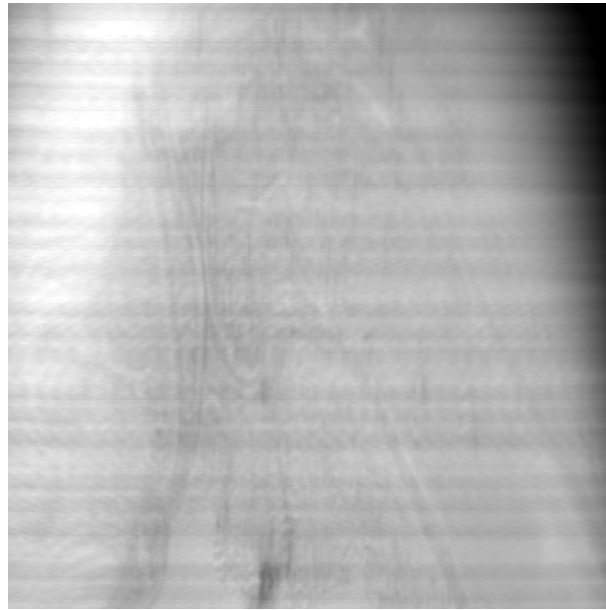


Figure 6.1.3: Cropped transmission reconstruction of sample AI-53 CL RGB. The grayscale modulation reveals internal morphological features of the stained mouse limb section.

7 | Conclusions and Future Perspectives

7.1. Summary of the Work

This thesis addressed the design, implementation, and experimental validation of the opto-mechanical scanning and acquisition backbone of a compact reflectance confocal microscope (RCM) prototype conceived for non-invasive, cellular-resolution imaging in dermatology. Starting from the clinical motivation and from a critical analysis of the state of the art, the work established the physical and engineering framework of RCM by detailing the confocal imaging principle, the wavelength–numerical-aperture trade-offs governing lateral and axial resolution, the endogenous sources of reflectance contrast in skin, and the fundamental constraints linking scanning strategy, pixel dwell time, and signal-to-noise ratio in video-rate operation. On this basis, an illumination and scanning architecture was designed around a high-speed raster generated by a rotating polygon mirror for the fast axis and a galvanometric mirror for the slow axis, combined with two properly conjugated $4-f$ relays to preserve pivot conjugation and deliver a collimated, two-axis scanned beam at the objective pupil while mitigating clipping and aberration growth at large deflection angles. The opto-mechanical realization included the selection of near-infrared illumination at 980 nm, the integration of commercial achromatic relay optics, and the fabrication of dedicated mechanical interfaces and custom mounts to ensure stable alignment and reproducible positioning. In parallel, the electronic and computational infrastructure required for deterministic temporal-to-spatial mapping was developed by leveraging a STEMLab 125-14 platform as a unified waveform-generation and acquisition node, implementing scanner drive signals, deep-memory data capture, and high-throughput data streaming to a host PC. A dedicated optical Start-Of-Scan (SOS) subsystem provided line-level timing references synchronized to the polygon facets, enabling robust line segmentation and frame reconstruction under non-ideal scan duty cycles. A host-side software pipeline was then implemented to manage data reception and storage, reconstruct images from the sampled detector waveform using the SOS timing markers and the slow-axis reference, and provide visualization tools, including automated cropping of the effective illuminated field of view to remove non-informative margins arising from facet transitions and scan-envelope truncation. To keep the experimental campaign aligned with the primary objective of implementing the most timing-critical and software-intensive subsystems, the prototype was aligned and validated in a transmission configuration up to the pupil-conjugate plane of the second relay, without the objective in place: in this controlled geometry, staged tests on empty-field scans, high-contrast objects, and a standardized USAF resolution target verified raster stability, polygon/galvo synchronization, acquisition bandwidth adequacy, and reconstruction correctness, while imaging experiments on heterogeneous biological tissue sections confirmed the robustness

of the end-to-end synchronization and reconstruction chain on realistic samples. Although a full epi-confocal alignment of the detection arm (pinhole centering, detector conjugation, and fine optimization of the return path) was outside the time frame of this work, the polarization-based beam routing and detection layout were mechanically integrated and preliminarily verified.

7.2. Limitations of the Current Prototype

Despite the successful validation of the scanning, synchronization, and reconstruction backbone, the present prototype still exhibits several limitations that constrain both quantitative image fidelity and the immediate transition to fully operational reflectance-confocal imaging. First, the transmission reconstructions of the USAF 1951 target reveal a field-dependent geometric distortion that becomes more evident toward the periphery of the illuminated scan envelope, as visible in the lettering and bar-pattern images, together with a progressive degradation in contrast and sharpness at large scan excursions. The dominant effect is consistent with a *barrel-type* distortion (negative distortion), which is expected when the image formation is based on a linear time-to-pixel mapping while the true angle-to-position transfer is intrinsically nonlinear (approximately $x \propto f \tan \theta$) in the absence of an f - θ scan lens. In the current optical train, the two 4 - f relays were designed primarily to preserve pivot conjugation and to deliver a collimated, two-axis scanned beam at the objective back-aperture plane, but they are not optimized to enforce an f - θ mapping or wide-field telecentricity. Consequently, the fast-axis polygon scan, although near-constant in angular velocity over the usable portion of a facet, does not translate into a strictly constant spot velocity at the sample (or validation) plane, and the resulting nonuniform magnification across the field manifests as distortion and spatial-frequency-dependent sampling errors in the reconstructed image. This limitation is compounded by scan-angle-dependent clipping and vignetting at the relay apertures and at the polygon facet transitions, which reduce the effective duty cycle of each line and impose the systematic use of cropping to isolate the useful field of view. From an opto-mechanical standpoint, the prototype also inherits practical constraints associated with the high density of components around the polygon scanner and with the use of custom 3D-printed mounts: the Start-Of-Scan optical subsystem could not be arranged to use the same polygon facet as the main illumination beam, which can introduce facet-dependent timing offsets and residual line-to-line registration errors, and the overall alignment remains sensitive to small angular and axial misplacements of relay lenses and scanner axes that become increasingly detrimental at large deflection angles. Finally, the source beam quality and conditioning represent an additional limitation for ultimate confocal performance: the measured ellipticity of the emitted beam and the lack of dedicated beam-shaping optics can lead to an anisotropic focal spot and imperfect objective overfilling, thereby reducing the effective numerical aperture and spatial resolution. Addressing these aspects is therefore required to transform the present proof-of-principle scanning platform into a geometrically accurate, diffraction-limited reflectance confocal microscope suitable for quantitative imaging.

7.3. Future Work and Perspectives

The results presented in this thesis establish a solid and experimentally validated platform for high-speed raster scanning and deterministic acquisition, and they naturally indicate a set of technical developments required to reach a fully operational, quantitatively reliable reflectance confocal microscope. The first and most immediate step is the completion of the epi-detection branch alignment: this includes enforcing precise conjugation between the illuminated focal volume and the detection pinhole plane, centering the pinhole on the optical axis, and optimizing the return-path relay so that the backscattered field is imaged onto the active area of the photodetector with maximal etendue matching while minimizing stray light. In practice, this requires iterative alignment procedures to converge toward the confocal condition that provides effective out-of-focus background rejection and optical sectioning. Once the high-NA objective is integrated and the confocal detection arm is tuned, a systematic characterization campaign should be carried out to quantify lateral and axial resolution, contrast, signal-to-noise ratio, and field-dependent performance, including the measurement of the point spread function and modulation transfer function as a function of scan position and depth in tissue-mimicking phantoms, together with an assessment of speckle statistics. In parallel, optical refinements should target the reduction of aberrations and geometric distortion: tighter control of relay centering and spacing and the introduction of scan optics that enforce an f - θ mapping would increase the usable field of view and restore spatially uniform sampling. Beam-conditioning optics, including circularization and controlled objective overfilling, would further improve the effective numerical aperture and mitigate anisotropic resolution. On the instrumentation side, further exploiting the STEMLab-based architecture can enable higher acquisition throughput and improved real-time interaction: implementing a low-latency streaming pipeline, GPU-accelerated reconstruction, and a dedicated graphical interface would permit live video-rate imaging with higher effective frame rates, dynamic adjustment of the scan parameters, and online quality metrics (e.g., contrast, saturation, and motion indicators), which are essential for user-guided focusing and for operation on living subjects. Similarly, more advanced synchronization strategies, including facet-specific timing calibration and line-by-line phase correction, would improve long-term stability and reduce residual registration errors. Finally, once stable reflectance-confocal imaging is achieved, the platform provides a basis for clinically relevant extensions such as depth-stack acquisition for 3D visualization, mosaicking for wide-area mapping, and quantitative contrast analysis in skin based on intrinsic reflectance, with the long-term perspective of integrating the prototype into a compact, robust, and reproducible instrument that balances optical performance, cost, and usability for translation into dermatological applications.

A | Datasheets

A.1. Objective lens (Nikon CFI Apo NIR 40× W)

Datasheet (online): Nikon CFI Apo NIR 40× W.

URL: https://www.microscope.healthcare.nikon.com/it_EU/products/optics/selector/compare/-1954

A.2. Laser source (CNI MDL-III-980L)

Datasheet (online): CNI MDL-III-980L.

URL: <https://www.cnilaser.com/PDF/MDL-III-980L.pdf>

A.3. Fast scanner (Polygon mirror Gecko-45-HSS)

Datasheet (online): Gecko-45-HSS.

URL: <https://precisionlaserscanning.com/wp-content/uploads/2020/06/GECKO-45-HSS-BROCHURE-25MAY20.pdf>

A.4. Slow scanner (Thorlabs GVS001)

Datasheet / manual (online): Thorlabs GVS001.

URL: <https://www.thorlabs.com/item/GVS001>

A.5. Achromatic doublets (Thorlabs)

Product page (online): Thorlabs Mounted Achromatic Doublets (AR 400–1100 nm).

URL: <https://www.thorlabs.com/mounted-achromatic-doublets-ar-coated-400---1100-nm>

A.6. Polarizing beam splitter (Thorlabs PBS122)

Product page (online): Thorlabs Broadband Polarizing Beamsplitter Cubes (PBS122 series).

URL: <https://www.thorlabs.com/broadband-polarizing-beamsplitter-cubes>

A.7. Quarter-wave plate (Thorlabs WPQ10ME-980)

Product page (online): Thorlabs Polymer Zero-Order Quarter-Wave Plates.

URL: <https://www.thorlabs.com/polymer-zero-order-quarter-wave-plates>

A.8. Pinhole (Thorlabs)

Product page (online): Thorlabs Circular Precision Pinholes (Vacuum Compatible).

URL: <https://www.thorlabs.com/circular-precision-pinhole-stainless-steel-foils-vacuum-compatible>

A.9. Detector module (Analog Modules 712B-2 APD)

Datasheet (online): Analog Modules 712B / 712B-2.

URL: <https://analogmodules.com/wp-content/uploads/712B.pdf>

A.10. Motorized linear stage (Zaber LSA10A-T4A)

Datasheet (online): Zaber LSA10A-T4A.

URL: <https://www.zaber.com/api/assets/LSA10A-T4A-Datasheet.pdf>

A.11. Motor controller (Zaber X-MCC1)

Datasheet (online): Zaber X-MCC1.

URL: <https://www.zaber.com/api/assets/X-MCC1-Datasheet.pdf>

A.12. DAQ (STEMlab 125-14 RedPitaya)

Datasheet (online): RedPitaya STEMlab 125-14.

URL: <https://redpitaya.com/stemlab-125-14/>

A.13. Start-of-Scan (SOS) Kit Precision Laser Scanning

Product page (online): Precision Laser Scanning SOS KIT.

URL: <https://precisionlaserscanning.com/wp-content/uploads/2019/07/miniSOS-KIT-BROCHURE-13jJUL19.pdf>

A.14. Photodetector PDA36A2 (Thorlabs)

Product page (online): Thorlabs PDA36A2 Detector.

URL: <https://www.thorlabs.com/item/PDA36A2>

A.15. Oscilloscope (Teledyne LeCroy HDO4000A Series)

Product page (online): Teledyne LeCroy HDO4000A Oscilloscope.

URL: <https://cdn.teledynelecroy.com/files/pdf/hdo4000a-oscilloscopes-datasheet.pdf>

A.16. CMOS Camera (Thorlabs DCC1545M)

Product page (online): Thorlabs DCC1545M CMOS Camera.

URL: <https://www.thorlabs.com/item/DCC1545M>

A.17. USAF Target

Product page (online): Edmund Optics USAF target.

URL: <https://www.edmundoptics.eu/f/1951-usaf-glass-slide-resolution-targets/12064/>

B | Electronics Connections

B.1. Laser Head ↔ PSU-III-LED Controller

- Pin 1: LD+ Positive terminal for the laser diode power supply.
- Pin 2: LD+ Redundant positive terminal to ensure high current stability.
- Pin 3: CRYSTAL TEC - Negative drive line for the crystal thermoelectric cooler.
- Pin 4: NTC CRYSTAL SENSOR Input for the crystal temperature thermistor.
- Pin 5: CASE Chassis ground connection for electromagnetic shielding.
- Pin 6: INTERLOCK Safety loop signal; open circuit disables laser emission.
- Pin 7: NTC LD Input for the laser diode temperature thermistor.
- Pin 8: NTC LD Return path for the laser diode thermistor signal.
- Pin 9: LD- Negative terminal for the laser diode power supply.
- Pin 10: LD- Redundant negative terminal for current return.
- Pin 11: CRYSTAL TEC+ Positive drive line for the crystal thermoelectric cooler.
- Pin 12: NTC CRYSTAL SENSOR RETURN Return path for the crystal thermistor.
- Pin 13: GND Common system ground.
- Pin 14: LD TEC - Negative drive line for the laser diode thermoelectric cooler.
- Pin 15: LD TEC + Positive drive line for the laser diode thermoelectric cooler.

B.2. X-MCC1-KX13BF Controller ↔ LSAT104A-10 Translation Stage

- Pin 1: A+ PHASE Positive drive signal for motor phase A.
- Pin 2: A- PHASE Negative drive signal for motor phase A.
- Pin 3: B+ PHASE Positive drive signal for motor phase B.
- Pin 4: B- PHASE Negative drive signal for motor phase B.
- Pin 5: GND Electrical ground reference.
- Pin 6: LIMIT/HOME Input signal from the home and travel limit sensors.
- Pin 7: +5 V DC Auxiliary power supply for stage-mounted electronics.
- Pin 8: ID Hardware identification signal for automatic stage recognition.

- Pin 9: ENCODER A Incremental encoder quadrature signal channel A.
- Pin 10: ENCODER A* Differential complement of encoder channel A.
- Pin 11: ENCODER B Incremental encoder quadrature signal channel B.
- Pin 12: ENCODER B* Differential complement of encoder channel B.
- Pin 13: ENCODER INDEX Absolute reference pulse (index) for zeroing.
- Pin 14: ENCODER INDEX* Differential complement of the index signal.
- Pin 15: SHIELD External cable shielding for EMI mitigation.

B.3. STEMLab 125-14 ↔ Amplifier Board

OUT 1: Polygon drive signal Analog control voltage for the polygon motor speed regulation via SMA interface.

OUT 2: Galvo drive signal Analog control voltage for the galvanometric mirror positioning via SMA interface.

GND Common signal ground reference between the DAQ system and the amplifier circuitry.

EXTERNAL ENABLE Digital logic output from STEMLab to enable the polygonal mirror motor.

DRIVER STATUS Digital feedback signal from the amplifier board to the STEMLab for monitoring operational faults.

B.4. Galvo Driver ↔ Amplifier Board

Pin 1: SIGN+ Positive terminal of the differential analog command signal.

Pin 2: SIGN- Negative terminal of the differential analog command signal.

Pin 3: DRV STATUS Digital output indicating the operational and fault status of the driver.

Pin 4: EXT ENABLE External logic input used to enable or disable the driver output stage.

Pin 5: -12 V DC Negative analog power rail for internal signal processing circuitry.

Pin 6: +12 V DC Positive analog power rail for internal signal processing circuitry.

Pin 7: GND Power ground reference for current return.

Pin 8: GND Signal ground reference for low-noise command stability.

B.5. Galvo Motor ↔ Galvo Driver

Pin 1: MOTOR COIL + Positive drive current for the galvanometer actuator coil.

Pin 2: MOTOR COIL - Negative drive current for the galvanometer actuator coil.

Pin 3: +15 V DC Positive supply for the integrated optical position detector.

Pin 4: -15 V DC Negative supply for the integrated optical position detector.

Pin 5: GND Common ground for position feedback and sensor circuitry.

Pin 6: MIRROR POSITION Analog voltage signal representing the real-time angular position.

Pin 11: +5 V REF Precision reference voltage for the feedback sensor calibration.

Pin 12: GND Shielded ground reference for signal stability.

Pin 13: TEMPERATURE MONITOR Analog output for motor thermal supervision and protection.

Pin 15: SHIELD Grounded cable shield to minimize inductive noise and EMI.

B.6. Galvo Driver ↔ STEMLab 125-14

MIRROR POSITION Input signal to the DAQ for closed-loop monitoring via signal conditioning board.

GND Signal ground reference between the driver unit and the DAQ acquisition system.

B.7. Amplifier Board ↔ Rotating Polygonal Mirror

Pin 1: +24 V DC Main power supply rail for the brushless DC motor.

Pin 2: GND Power return path and common reference.

Pin 3: POLYGON MOTOR ENABLE Logic signal to initiate or halt the polygonal mirror rotation.

Pin 4: AT SPEED STATUS Feedback signal indicating the motor has reached the target angular frequency.

Pin 5: TTL EXTERNAL FREQUENCY REFERENCE External clock signal for phase-locked loop synchronization.

B.8. 521A-1 HV Power Supply ↔ 712B-2-DC Detector

+15 V DC Positive bias supply for the detector's integrated preamplifier.

-15 V DC Negative bias supply for the detector's integrated preamplifier.

HV OUTPUT High voltage bias required for the detector's active sensing element.

GND High voltage and signal ground reference for noise minimization.

B.9. 712B-2-DC Detector ↔ STEMLab 125-14

DETECTOR SIGNAL Analog voltage output proportional to the incident optical intensity.

B.10. SOS Detector ↔ STEMLab 125-14

SOS DETECTOR SIGNAL TTL pulse indicating the Start of Scan (SOS) synchronization event.

GND Signal ground reference for the synchronization pulse.

C | Software

The complete software developed for this thesis, including the C source code for the Red Pitaya (**Firmware/** folder), the Python acquisition server, and the visualization GUI (**Host/** folder), is available for consultation at the following GitHub repository:

Repository: [github.com/LeonardoFederighi/Reflectance-Confocal-Microscope-MD-Thesis-](https://github.com/LeonardoFederighi/Reflectance-Confocal-Microscope-MD-Thesis)

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