CompassLSM: axially swept light-sheet microscopy made simple

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Abstract: Axially swept light-sheet microscopy (ASLM) is an effective method of generating a uniform light sheet across a large field of view (FOV). However, current ASLM designs are more complicated than conventional light-sheet systems, limiting their adaptation in less experienced labs. By eliminating difficult-to-align components and reducing the total number of components, we show that high-performance ASLM can be accomplished much simpler than existing designs, requiring less expertise and effort to construct, align, and operate. Despite the high simplicity, our design achieved 3.5-µm uniform optical sectioning across a >6-mm FOV, surpassing existing light-sheet designs with similar optical sectioning. With well-corrected chromatic aberration, multi-channel fluorescence imaging can be performed without realignment. This manuscript provides a comprehensive tutorial on building the system and demonstrates the imaging performance with optically cleared whole-mount tissue samples.

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1. Introduction

With the emerging need for 3D imaging of whole-mount tissue samples, light-sheet fluorescence microscopy (LSFM, also called selective/single plane illumination microscopy (SPIM)) has become a popular technique in biomedical research [1–5]. The light-sheet microscope is an upgrade of the fluorescence wide-field microscope. Instead of using transmissive or epi-illumination, a sheet of light is used to illuminate a thin plane inside the sample. This configuration provides the optical sectioning advantage of confocal microscopes and the speed advantage of camera-based systems. As early as 1902, Zsigmondy et al. invented the first light-sheet microscope [6]. The light sheet was generated by projecting sunlight through a rectangular slit aperture using a finite conjugate microscope objective. Light scattered from colloidal gold was observed using a conventional wide-field microscope. The modern LSFM was first introduced by Voie et al. in 1992 as orthogonal-plane fluorescence optical sectioning (OPFOS) to create a 3D image of an optically cleared guinea pig cochlea [7]. The light sheet was generated by focusing a collimated laser beam using a cylindrical lens, which enabled 3D imaging using a digital microscope with a 10-µm lateral resolution, a 26-µm axial resolution, and a 1.5-mm field of view (FOV). In 2004, Huisken et al. further demonstrated the efficacy of LSFM on zebrafish and drosophila melanogaster, achieving up to 6-µm lateral resolution and 6-20-µm optical sectioning in a 2.5-mm FOV [8]. Since then, LSFM has rapidly evolved with growing needs for cutting-edge imaging performance (e.g., high speed, large FOV with high resolution, etc.) in biomedical research.

While some LSFM systems are commercially available, it is common for research groups to build customized light-sheet microscopes due to lower costs and more design flexibility [9–11]. Several research groups have also developed open-hardware LSFM projects such as OpenSPIM [9], allowing researchers to build their LSFM without extensive knowledge of optical engineering. However, compared to commercial systems, customized optical systems often require extra resources to build and maintain. It is still challenging for biomedical researchers to start a customized light-sheet project with limited expertise in optical engineering. Besides, simple
customized LSFM often uses Gaussian-beam-based illumination using cylindrical lens focusing. The optical sectioning resolution and FOV of the system are intrinsically limited.

A fundamental challenge for designing a simple Gaussian-based LSFM is simultaneously improving the axial resolution and the FOV. A physical tradeoff exists between the beam waist width and the Rayleigh length (or confocal parameter) with a focused Gaussian beam. While the beam waist determines the light-sheet thickness and the optical sectioning resolution, the Rayleigh length determines the effective FOV in the direction of the illumination propagation. Therefore, a simple Gaussian beam-based light-sheet microscope often suffers from either limited axial resolution or reduced FOV. Optical engineers have developed various methods to improve both axial resolution and FOV at the same time. For instance, scanned non-Gaussian beams such as Bessel beams and structured light demonstrated promising capability for generating a thinner sheet over a wider FOV in modern LSFM designs [14,15]. Still, the high design complexity and low availability of these advanced systems limit their use in a broader range of biomedical research applications.

A simpler LSFM design with good imaging performance would greatly benefit the biomedical research community. Therefore, we aim to develop a high-performance LSFM with a simple design. Among many LSFM designs, we found that the Axially Swept Light-sheet Microscope ASLM [12] and its descendants [10,13,16,17] demonstrated outstanding potential for high-performance, large-FOV imaging. ASLM works by synergistically combining two advanced LSFM design concepts. The first concept, extended focusing, refers to scanning the focus of a Gaussian light sheet along the optical axis [18]. The second concept, rolling shutter synchronization [19] or “confocal slit” detection [20,21], refers to using a rolling shutter camera to perform line-by-line scanning (see Fig. 1). The rolling shutter acts as a dynamic confocal slit rejecting regions with suboptimal illumination. By synchronizing the active rolling shutter to the swept light sheet’s best focus, optical sectioning remains thin and homogenous throughout the entire FOV.

Inspired by the ASLM-based mesoSPIM [10] and Design for Manufacturing and Assembly (DFM/A) principles [22], we simplified the ASLM design to improve assembly and alignment of the system. We named this system Compact Axially Swept Scanned Light Sheet Microscope (CompassLSM). CompassLSM features both an extremely lean structural design and superior imaging performance for large cleared tissue samples. To our knowledge, CompassLSM is the simplest and most compact design of ASLM, requiring the least effort to build. The module consists of less than 20 optical and optomechanical components (excluding screws), including a single customized CNC-machined platform with specialized mechanical features assisting fast and precise assembly. The straightforward design also enhanced the tolerance to optical misalignments by avoiding hard-to-align optical elements used in other ASLM systems (e.g., cylindrical lenses, aspherical lenses, relay lenses, and piezo mirrors [10,12,13,16]). It only takes minutes to assemble and align the device in less than ten steps, notably less than existing ASLM designs and requiring no advanced knowledge in optical alignment or prototyping. With a small footprint and a simple control scheme, the system can be easily built into existing fluorescence imaging systems with minimal effort. An achromatic design makes the system easily switchable between 480 nm and 650 nm illumination without reconfiguration. Approximately 3.5-um optical sectioning can be achieved over a >6-mm FOV using inexpensive detection optics that are widely and often readily available. While the system is more cost-effective (~$12,000) than commercial LSFM illuminators with similar specifications, the FOV is larger than commercial/customized systems with similar optical sectioning, making it an effective tool for imaging large, whole-mount tissue samples. This manuscript serves as a tutorial for designing and building such a system from scratch.
Fig. 1. The working principle of ASLM. The grids represent the array of the rolling shutter camera sensor. The blue (top left legend for colorblind) hyperbola represents a focused Gaussian beam in the sample plane. The red (top middle) rectangles represent the focal region (e.g., Rayleigh range) of the Gaussian beam. The purple (top right) represents the active lines in the rolling shutter image sensor. $t_0 < t_1 < t_2$ represents 3 discrete time points during one image acquisition. a) A still Gaussian beam in the sample plane: a short narrow band of sample is in good focus. b) Virtual/digitally-scanned lightsheet: by quickly scanning the Gaussian beam in the lateral direction using a scan mirror, a short but wide band of sample is in good focus. c) Rolling shutter sensor: only a few consecutive lines on the sensor are under exposure at a time. d) Axially-swept light sheet: the in-focus position of the virtual light sheet is scanned with an optical configuration such as an electrically tunable lens (ETL), and the location of the in-focus band is synchronized with the active exposing lines in the image sensor. e) Volumetric representation of a-d), demonstrating the working principle of ASLM. f-h) Selected optical designs of ASLM from previous publication [10,12,13]. i) Optical design of CompassLSM, showing a significantly simplified structure. Abbreviation: CB-collimated beam, HWP-half wave plate, CL-cylindrical lens, L-lens (spherical), SL-scan lens, TL-tube lens, PBS-polarization beam splitter, QWP-quarter wave plate, MO-microscope objective, 4f-relay lens pair, ETL-electrically turntable lens, and RCO-reversed camera objective. An animated version of a-e is shown in Visualization 1.
2. System design

2.1. Design overview

We started the CompassLSM design process by defining three primary functional components for a scanned ASLM light sheet generator. First, a beam collimator is required to create a diffraction-limited spot or sheet. Second, an optical scanning and focusing system is required to generate a thin scanned light sheet. Finally, a dynamic focusing device is required to sweep the scanned sheet along the optical axis. In addition, we studied the technical specifications of existing LSFM devices used for whole-mount cleared tissue imaging. We found that most commercial light-sheet microscopes for cleared tissue imaging (e.g., LaVision Ultramicroscope II, PhaseView Alpha3, Lifecanvas SmartSPIM, Zeiss Z1, etc.) had a FOV greater than 1 mm (10×), optical sectioning between 1–12 μm, and a working distance greater than 15 mm. These specifications guided our design objectives for the CompassLSM and helped us identify a simple configuration for each of the components. In addition, we designed a central mounting platform to achieve easy assembly and alignment. Based on these design inputs, we identified 7 design considerations (2.2–2.8) and built our system with two submodules (Collimator module and Scan module, Fig. 2). The assembly procedure of the system is also shown in Fig. 2. All components of the system, part numbers, and cost are listed in Fig. S1.

2.2. Beam collimator design

A clean, collimated beam is essential to light sheet generation. We chose to use single-mode fiber (SMF)-coupled lasers as the light source. An SMF-coupled laser device is more costly than a free-space laser, but it is highly beneficial for a modular LSFM generator. SMF-coupling eliminates the need for mechanical-pinhole-based spatial filtering of the laser, reducing the optical path length and alignment requirements. The laser becomes a modular device that can be maintained separately from the microscope and shared between multiple imaging systems. The single-mode output from the SMF is readily collimated with a single lens (e.g., a collimator). The collimated beam diameter is also easily adjusted by changing the focal length of the collimator. Among many collimator design options, we found that an achromatic doublet outperformed simple singlet lenses, aspheric lenses, and aspherized achromatic lenses. An achromatic doublet has sufficient numerical aperture (NA) for an SMF output (e.g., NA < 0.2). It is also insensitive to chromatic aberration and minor misalignment due to decentering and tilting, requiring minimal effort to align. Thus, an SMF-coupled laser and an achromatic doublet are used in our design to create the collimated beam.

Any SMF-coupled laser in the visible wavelength range can be used in CompassLSM. In this study, we primarily use a commercial SMF-coupled multi-channel laser combiner (Omicron SOLE-6 laser engine with 405/488/561/647 nm diode/DYSS lasers). We also tested simple SMF-coupled lasers built from bare single-mode laser diodes (e.g., Sharp GH04850B2G, USHIO HL63603TG, etc.) and simple constant current drivers (e.g., Wavelength FL591FL, Thorlabs LDC205C, etc.), which demonstrated no noticeable performance difference. The output beam from the SMF is collimated with an achromatic doublet (Thorlabs, AC254-050-A, f = 50 mm). The distance between the SMF and the collimating lens can be adjusted with a screw-based lens tube mechanism. The collimation can be determined by either minimizing the spot size at a distance or using a shearing interferometer. With the SMF output NA around 0.12, a high-quality ~12-mm diameter (1/e2) Gaussian beam can be produced. A mechanical iris diaphragm (Thorlabs SM11D12C) can be used to adjust the diameter of the collimated beam. The finite aperture may introduce side lobes in the focused beam, but it should minimally affect the optical sectioning capability because the majority of the power is confined in the major lobe.
Fig. 2. Schematic showing the components and assembly/alignment process of the CompassLFM illuminator. All part numbers are for Thorlabs unless otherwise specified.

a) Explosion diagram of the upper collimation module, comprising 10 components: 1. Single-mode FC/PC fiber input; 2. S1FC fiber adapter; 3. SM1V10 Ø1” adjustable lens tube; 4. AC254-050-A Ø1”−50 mm achromatic doublet; 5. SM1L10 Ø1” lens tube; 6. SM1D12C iris diaphragm; 7. SM1SH1 beam shutter (optional, can be inserted anywhere between 2–8); 8. KCB1C cage mirror mounts (2 pieces); 9. BB1-E02 dielectric mirrors (2 pieces); and 10. ER05 cage rods. b) Assembly of the cage-based fold mirrors. c) Assembly of the fiber-output laser collimator. Screw in the lens tube and minimize the far-field spot size. d) Assembly of the collimator module; e) Adjust the fold mirrors to center the collimated beam using two DG10-1500-H1-MD alignment disks and SM1E60 extension tubes. f) Explosion diagram of the lower scan module, comprising another 8 components: 11. SPT1 slip plate; 12. SM1 to C-mount thread adapter (SM1A10 or SM1A39); 13. ETL (Optotune EL-16-40-TC, Edmund Optics #33-494/88-940/88-941); 14. 6-mm 2-axis galvo scanner (Cambridge 6215H); 15. Objective lens (Olympus XLFLUOR 4x/340 NA 0.28); 16. PT1 1” manual stage for light sheet focus adjustment; 17. PT1 or PT1A 1” manual stage for light sheet location adjustment; and 18. customized aluminum mounting platform. All parts are assembled as shown by the arrows. g) Alignment of parts 11, 14, 16 & 18, with dotted lines indicating coplanar alignment. h) Alignment between parts 14, 15 & 18, with dotted lines indicating coplanar alignment. i) Final assembly of the collimating module and the scan module. Control signals for optoelectronics are also indicated in the diagram. j) Final assembly accomplished by mounting part 8 against 18. 8 is secured to 11 by the cage system.
2.3. Lateral scanning and focusing system

The “scanned light-sheet” functionality (Fig. 1(b)) requires a mechanical scanning device and an optical focusing device. It is often achieved through a 4-element infinity-corrected laser scanning microscope configuration (e.g., galvanometer scanner (galvo) + scan lens + tube lens + objective lens) in many light-sheet microscope designs [20,23,24]. However, when submicron resolution is not required, this configuration can be reduced into a simple 2-element design (e.g., galvo + scan lens) - a common design used in low-resolution applications such as laser engraving [25], optical coherence tomography [26], and selective laser sintering 3D printing [27]. In mesoSPIM [10], an inverted consumer camera objective was repurposed as the scan lens. Here, we found that a smaller microscope objective lens (Olympus XLFLUOR 4x/0.28) can be directly used as a telecentric scan lens in a 2-element scanning system. This medium NA large FOV objective is widely used in various LSFM designs, such as the COLM light sheet for CLARITY imaging [23,28].

The back focal plane of this objective is ∼14 mm away from the end of the lens barrel, measured with a collimated laser beam. The center axis of the second mirror of a galvo scan head with a 6-mm aperture (Cambridge Technology, 6215H) is ∼11.5 mm from the surface of the standard mounting block. By installing the objective against the mounting block, the back focal plane of the objective is placed between the galvos, enabling quasi-telecentric scanning [29]. As perfect telecentric scanning is not essential in this application, small distance variations between the galvo and the focusing objective do not significantly affect the performance of the system. We also note that small decenter and tilt misalignments are well tolerated by the objective because only a fraction of the objective’s aperture (e.g., 6 mm out of 25 mm) is used in this application. Therefore, the combination of the objective lens and galvo is used to generate a scanned light sheet. With a ∼6-mm beam diameter and a ∼28.5-mm working distance, the NA of the system is ∼0.1, corresponding to ∼3-µm resolution (light sheet thickness) at a 500-nm wavelength.

2.4. Axially swept light-sheet

The “axially swept” functionality requires a dynamic focusing device. The original ASLM [12,30] by Fiolka’s group has a relatively high design complexity because a low-aberration remote focusing mechanism [31] is implemented in the illumination setup (Fig. 1(f)). High performance is achieved at the cost of more than ten major optical components and a sophisticated alignment procedure. By replacing the remote focusing with an electrically tunable lens (ETL) [13], Hedde et al. simplified the design to only five major optical elements (Fig. 1(g)). Although the optical sectioning capability is reduced from the submicron level to above a micrometer, the swept light sheet is still significantly thinner than a low-NA Gaussian beam to achieve the same FOV. In mesoSPIM [10], Voigt et al. modified the ASLM design by replacing the cylindrical lens with the digitally scanned light-sheet mechanism [32]. An ETL was placed at the conjugate pupil plane of the focusing lens using a 4-f relay lens pair to ensure telecentric scanning (Fig. 1(h)). Although the optical sectioning is reduced to >6 µm, over 10 mm FOV is achieved with the mesoSPIM. To further simplify and miniaturize the design, we implemented an ETL (Optotune, EL-16-40-TC-VIS-5D-C) in the optical path closely before the galvos without relay optics (Fig. 1(i)). We determined that even though the relay optics improves telecentricity, the improvement was negligible in this application (see Section 4.9.).

The ETL requires a finer alignment than other elements in this design. First, it needs to be installed in the vertical direction to prevent wavefront distortion due to gravity. Second, the ETL is also sensitive to decenter misalignment, potentially resulting in beam decentering and higher-order optical aberrations and causing varied spot sizes during axial sweeping. To ensure the ETL’s alignment in the system, we implemented a fold mirror pair (Thorlabs, KCB1C and BB1-E02) after the collimated beam to center the collimated beam through the ETL. The alignment of the beam is verified by passing the beam through the center holes in the two
alignment disks (Thorlabs, DG10-1500-H1-MD) separated by >100 mm using a long lens tube. As an alternative to the fold mirror pair, a kinematic optical mount with 5 axes of freedom (e.g., Thorlabs, KC1-S) could also be used to center the beam. It could further reduce the cost and size of the design, but the alignment procedure would be more complicated and long-term stability less certain.

2.5. Central mounting platform

The central mounting block (CMB) of the 3 submodules is designed using SolidWorks and fabricated with 6061 aluminum using an online CNC-machining service (e.g., 3DHubs.com). The CMB is designed with features to assist the alignment process. The design tolerance of the CMB is ±0.005", but the final machining error is less than 0.001". The alignment of the objective and the galvo is directly designed within the CMB. The objective is wrapped with 0.005"-thick Teflon tape (McMaster-Carr, 6305A44) to fill the gap between the objective and the mounting slot created by the tolerance offset. The lens is then secured with set screws. Two surfaces of the galvo mount are aligned to two surfaces of the CMB (Fig. 2(h)). The ETL is attached to the CMB with an off-the-shelf slip plate optical mount (Thorlabs, SPT1), which also has 2 perpendicular surfaces aligned to the CMB (Fig. 2(g)). The XY-slip plate alignment is intended for compensating for the potential large machining error of the CMB, which can be measured with a micrometer. The illumination module and the fold mirrors are assembled in a compact optical cage system and attached to the slip plate. The fold mirror mount also has a surface coplanar with the CMB. The CMB is mounted on a manual 2-axis translational stage (Thorlabs, PT1&PT1A) for axial and horizontal alignment of the light sheet. The mechanical drawing of the CMB is shown in Fig. S2.

2.6. Detection optics and other components

CompassLSM is designed to be compatible with a wide variety of detection configurations. The minimum requirement of the detection system is a simple wide-field fluorescence microscope. In principle, the CompassLSM can be implemented in any wide-field microscope or macroscope with sufficient mechanical clearance to fit the illuminator and to generate a light sheet at the focal plane. In our primary setup, the customized microscope is assembled with an infinity-corrected microscope objective, a motorized filter wheel (Zaber, X-FWR-E), a tube lens (Thorlabs, TTL 200), a scientific CMOS camera (Hamamatsu, ORCA-Flash4.0 V3), a CNC-machined sample chamber, and a 3-axis translational stage (Physik Instrumente, M-112.1DG1). The microscope is assembled in an optical rail system with off-the-shelf optomechanics. We also tested a range of detection objectives in our system, including macroscope objectives (Olympus MVX PLAPO 1×/0.25 and MVX PLAPO 2XC/0.5), a low-cost, low-magnification air objective (Olympus UPlanFL 4×/0.13), and a high-NA 10× immersion objective (Nikon CFI Plan Apo 10XC/0.5). The lateral resolution of the system is sampling-limited in all cases. Despite the cost, quality, and optical aberrations, all tested objectives lenses generated promising results. For easy light-sheet-camera synchronization, a rolling-shutter camera sensor with the adjustable rolling shutter line speed is recommended to facilitate ASLM. As a side note, the design can potentially work with a conventional rolling-shutter camera sensor without an adjustable rolling shutter line speed, but it will be much more difficult to align the system with different imaging configurations (e.g., exposure time, binning, etc.). With 2 axes of galvo scanning, the system can create the light sheet at any orientation (e.g., vertical, horizontal, or arbitrary). It can also translate the light sheet along the detection optical axis, facilitating volume scanning without moving the sample.

2.7. Electronics and system control

The CompassLSM only requires a few simple low-speed analog signals to control. No programming is required to set up the system. A detailed explanation of the control signals is
described in Fig. S3. In brief, the minimum requirements are 1) a TTL pulse train to trigger the camera (frequency: frame rate (< 20 Hz)), 2) a triangle or sinusoidal wave to control the ETL (frequency: frame rate, amplitude: adjusted based on the FOV (< 3.3 V, 10 mV resolution)), and 3) a high-frequency triangle or sinusoidal wave to control the galvo (frequency: adjusted based on the camera exposure (200–1000 Hz), amplitude: adjusted based on the FOV (< 3.3 V, 10 mV resolution)). The camera trigger and the ETL driving signals are synchronized with an empirically determined phase delay (5-ms resolution). The galvo control signal does not need to be synchronized to the other signals. An optional square wave (frequency at frame rate, 60% duty cycle) can be implemented to control the laser output and reduce photobleaching. A PCIe DAQ card with 4 analog outputs is used as a function generator (National Instrument, PCIe-6363) is used in our setup. A LabView interface is used to control the DAQ. In principle, any 4-channel analog output device with simple waveform generation (peak voltage: > 3.3 V, resolution: > 10bit, bandwidth: > 10kHz) and adjustable phase delay can be used as a driver for CompassLSM. The microscope, translational stage, motorized filter wheel, and digital beam shutter (e.g., global laser switch) are controlled and coordinated by the open-source Micromanager software. The laser is controlled by the OEM software via serial communication. In theory, as long as the camera can be triggered by an external trigger signal and the microscope software can facilitate Z-stacking data acquisition, any wide-field fluorescence microscope and control software can be used with CompassLSM. A detailed alignment procedure of the system is shown in Fig. S4.

3. Experimental methods

3.1. Sample preparation

All rodent tissue samples were collected from discarded tissue samples of unrelated studies with IACUC approval. The rodents were euthanized with 4% paraformaldehyde (PFA, Electron Microscopy Sciences, 15710) transcardiac perfusion fixation under isoflurane or Avertin (2.5% tribromoethanol) anesthesia. The tissue samples were fixed in excess 4% PFA for an additional 2-4 h at room temperature, followed by 3 rounds of washes in phosphate-buffered saline (PBS, > 30 min each for all washes in this section). The quail embryo samples were prepared by incubating fertilized quail eggs at 37 °C for 5-6 days. The embryo samples were fixed in excess 4% PFA at 4 °C overnight, followed by 3 rounds of washes in PBS. All samples were stored at 4°C for up to 2 months. For immunohistochemistry staining, the samples were first bleached in excess 80% v/v methanol with 6% v/v hydrogen peroxide and 14% v/v water overnight at room temperature. The samples were then washed 3 times in PBS with 1% Triton X-100 (PBST). The samples were stained in a staining buffer (PBST + 3% w/v bovine serum albumin + 0.05% w/v sodium azide + 0.5% v/v antibody + optional 0.0001-0.001% w/v DAPI or propidium iodide) for up to 3 days at 37 °C. The samples were then washed 3 times in excess PBST at 37 °C. The secondary antibody staining was performed following the same procedure. The stained samples were stored at 4°C for up to 2 weeks. For optical clearing, the sample was cleared with several optical clearing methods (CUBIC [33], LIMPID (lipid-preserving index matching for prolonged imaging depth) [34], and ECI [35,36]) with refractive indices ranging from 1.46 to 1.55 [33–36]. The samples were mounted in quartz cuvettes filled with index matching solutions for imaging. The quartz cuvette is mechanically attached to a 3-axis stepper motor translational stage to facilitate stacking and stitching. During imaging, the cuvette is immersed in a customized imaging chamber with 3 clear windows. More detailed information about sample preparation for each dataset is discussed in the corresponding figure caption.

3.2. Data processing

Images presented in this manuscript were saved in 16-bit Open Microscopy Tiff format. In brief, basic image processing (e.g., denoising, downsampling, Z-intensity correction, etc.) and
rendering were performed in FIJI [37] and Amira (ThermoFisher). Video renderings of the data were generated with Amira and Imaris (Bitplane). 2D slices and maximum intensity projections were generated with FIJI. Advanced image processing and system characterization (e.g., beam waist Gaussian fitting) were performed in Matlab. All wide-field 2D renderings of the data are linearly stretched and downsampled for display on the screen. For 3D rendering, the images were processed with median and Gaussian filters to reduce high-frequency noise. The image backgrounds were predicted with sliding paraboloids (50–200 pixels) and subtracted from the original images. For volume rendering of large datasets, the data were downsampled to < 50GB or saved in pyramidal large data formats (e.g., HDF5). Downsampling was performed with 2×2 binning. Image stitching was performed with BigStitcher [38]. Some rendered images were color inverted, and the hue was shifted by 0.5 (0–1 scale) to improve the visual contrast.

4. Results and discussion

4.1. Mouse eye imaging

To demonstrate the large FOV of the design, we imaged a whole-mount mouse eye using CompassLSM (Fig. 3). The effective FOV of the system using a 1× macro objective is 5.8 mm for detection. With 2×2 binning (downsampling), a 5.7-μm isotropic sampling-limited resolution is achieved. The resolution is sufficient to resolve sparsely distributed cell nuclei in the cornea, lens, connective and muscular tissues. The 488-nm autofluorescence can be effectively used as a structural tissue label. Uniform optical sectioning is achieved throughout the entire FOV. Similar image quality is achieved in both lateral and axial planes. Additional eye images comparing regular Gaussian beams and ASLM are shown in Fig. S5.

4.2. Mouse pancreas imaging

To demonstrate the optical sectioning capability of the system over a large FOV, we imaged a whole-mount mouse pancreas stained with NeuN-Alexa Fluor 555 antibody (Sigma-Aldrich, MAB377A5, Fig. 4), which labels individual neuronal cell bodies. With a low NA 4× microscope objective, the entire mouse pancreas can be acquired in 2 image stacks. Each image stack has a 2.9-mm lateral FOV with 1.43 μm sampling. The image was taken with 2.85-μm z-sampling. Individual NeuN expressions can be resolved across the entire pancreas.

4.3. Mouse brain imaging

To demonstrate the ASLM functionality of the system, we compared the images of a whole-mount Thy1-GFP mouse brain with ASLM enabled and disabled. The distinct differences between the images are shown in Fig. 5(a)&(b). With ASLM disabled, the neural fiber structures are only resolved in < 15% of the entire FOV (Fig. 5(a)). The image quality, especially the axial resolution, significantly worsens with distance from the best focus. By enabling ASLM, fine neural dendritic structures are well-resolved across the entire 3×3×7-mm FOV. In addition, we imaged the same type of sample using a 10× multi-immersion objective Fig. 5(c)&(d). As shown in the figure, fine neural structures such as synaptic boutons and dendritic spines can be resolved in a 1.33×1.33×4.5-mm FOV with 0.65-μm lateral sampling. An entire mouse brain can be imaged by stitching multiple volumes, as shown in Visualization 5.

4.4. Mouse lung imaging

To showcase the multi-wavelength performance of the system, we imaged a mouse lung using 4 different excitation wavelengths (405, 488, 561, and 647 nm) across the visible spectrum (Fig. 6). The DAPI and autofluorescence images from each channel were recorded sequentially. All channels are well-aligned with each other at the pixel level across a 5.7-mm lateral FOV without
Fig. 3. Image of a mouse eye acquired using CompassLSM. The tissue was fixed with 4% PFA, beached with H2O2, stained with DAPI, cleared with LIMPID, and imaged with CompassLSM with a 1× macro objective (Olympus MVX PLAPO 1×/0.25). The setup has a 5.8-mm FOV with 2.85 µm lateral sampling. The image was taken with a 5.7-µm axial step size. Two excitation channels (405 nm for DAPI and 488 nm for autofluorescence) were captured sequentially. a) 3D rendering of the image of 2 channels combined. Individual extraocular muscular cell nuclei (DAPI) and muscle fibers (autofluorescence) are resolved. b) An XY slice in the middle of the volume. c) A YZ slice in the middle of the volume. A close-up view of the boxed region is shown on the right for each panel. The scale bars represent 1 mm in all images. XY represents the focal plane of the detection optics. A rendered video is shown in Visualization 2.

post-processing image registration. Fine features such as collapsed alveoli and valve structures are resolved several millimeters deep into the dense tissue.

4.5. Mouse intestine imaging

Most data shown above were taken with refractive index matching to ~1.47. To demonstrate the efficacy of the system over a large range of refractive indices, we imaged a section of mouse small intestine cleared with ethyl-cinnamate (ECi, refractive index 1.558) [36]. The volume rendering and a 2D slice of the mouse small intestine are shown in Fig. S9 and Visualization 7. As shown in the figure, propidium iodide-stained cell nuclei can be resolved through the entire volume with high refractive index optical clearing.

4.6. Quail embryo imaging

To demonstrate the overall performance and versatility of the system in challenging conditions, we imaged E5-6 whole mount quail embryos staining with TuJ1 and smooth muscle actin antibodies. Using a low-cost, low-NA 4× detection objective, it is possible to imaging across the entire quail embryo (~10 mm). Two channels of data can be acquired sequentially without post-alignment in
Fig. 4. Image of a mouse pancreas acquired using CompassLSM. The tissue was perfused with Lectin-Alexa Fluor 594, fixed with 4% PFA, bleached with hydrogen peroxide, stained with NeuN-Alexa Fluor 555 antibody, cleared with LIMPID, and imaged with CompassLSM with a low NA 4× microscope objective (Olympus UPlanFL 4×/0.13) in the detection path. The setup has a 2.9-mm FOV with 1.43 µm lateral sampling. The image was taken with a 2.85 µm axial step size. Two volumes were taken and stitched together. a) 3D rendering of the data showing uniform intensity across the entire sample. b) 3D rendering of the data with an XZ-slicing around the middle of the y dimension. The cutting surface is shown in linear grayscale. c) Zoom-in of the sub volume in b (orange boxed region), showing individual NeuN-positive cell nuclei on the block faces. d) Top half of the pancreas displayed as a maximum intensity projection with the depth color encoded (XZ plane). A zoom-in region of the image (orange box) is shown on the right. e) A zoom-in of a single positive cell in c (orange box, XZ plane), showing the spatial distribution of the signal for an individual cell body in this configuration (raw data with only linear stretch). f) 1D Plot of the normalized pixel values along the dotted line in e. The Gaussian fit of the data (red curve) has an 11.11 µm FWHM. (Note: cell nuclei are not limited diffraction spots) The peak pixel value is about twice above the background compared to the neighboring pixels. The scale bars represent 1 mm in all images. Depth (color) and pixel value are shown in the top left corner of d. XY represents the focal plane of the detection optics.
Fig. 5. Image of a Thy1-GFP mouse brain acquired using CompassLSM. The tissue was fixed and cleared with LIMPID. The top two datasets (a&b) were imaged with CompassLSM with a 2× macro objective (Olympus MVX PLAPO 2XC/0.5) in the detection path. The setup has a 2.95-mm lateral FOV with 1.44 μm lateral and 2.85 μm axial sampling. a) Image acquired without ASLM. b) Image acquired with ASLM enabled. Images are displayed as a maximum intensity projection with the depth color encoded (XZ plane). Zoom-in images from 5 regions across the XZ projections are displayed on the right of each image, showing the benefit of ASLM. The scale bar represents 1 mm for the images. Depth (color) and pixel value are shown in the top right corner. The bottom dataset (c&d) was imaged with a 10× immersion objective (Nikon CFI Plan Apo 10XC Glyc). 1.33 × 1.33 × 4.5 mm FOV with 0.65 μm lateral sampling can be achieved with this setup. The axial imaging depth is limited by the working distance of the objective.  c) Color-encoded-depth maximum intensity projection through the entire volume (XZ plane). d) 10-μm maximum intensity projections of lateral planes at 5 different imaging depths. Zoom-in views of the boxed regions are shown next to the corresponding image. The scale bars represent 100 μm. The arrows are pointing to fine neuronal structures such as boutons and spines. XY represents the focal plane of the detection optics. A rendered video of this dataset is shown in Visualization 3 and Visualization 4.
Fig. 6. Image of a mouse lung acquired using CompassLSM. The tissue was fixed, beached, DAPI stained, cleared with CUBIC, and imaged with CompassLSM with a 1x macro objective (Olympus MVXPLAPO 1×/0.25) in the detection path. The setup has a 5.8-mm lateral FOV with 2.85 µm lateral and 5.7 µm axial sampling. 4 excitation channels (405 nm for DAPI and 488, 561 & 647 nm for autofluorescence) were captured sequentially. a) 3D rendering a small portion of the data (561-nm autofluorescence) with some transparency. b) Solid 3D rendering of the same volume without transparency. Fine structures of individual alveoli are clearly resolved as shown in the zoomed in orange boxed region. c) A lateral (XY) 2D slice showing the multi-wavelength performance of the system. The orange boxed region is displayed to the right (rotated 90 degrees) with differing channels or combinations sequentially displayed along the tissue section to demonstrate the alignment of the channels. d) A medial (XZ) 2D slice showing the multi-wavelength performance of the system. The orange boxed region is displayed to the right with differing channels or combinations sequentially displayed along the tissue section to demonstrate the alignment of the channels. The scale bars represent 1 mm in all images. XY represents the focal plane of the detection optics. Zoom-in images of c and d are shown in Fig. S6&S7. Additional characterization of the multi-wavelength performance of the system is shown in Fig. S8. A rendered video of this dataset is shown in Visualization 6.
Fig. 7. Images of quail embryos acquired using CompassLSM. a) A Z-stack of an E5 quail embryo fixed, bleached with hydrogen peroxide, stained with Tuj1 and smooth muscle actin antibodies, cleared, and imaged with a low NA 4× microscope objective (Olympus UPlanFL 4×/0.13) in the detection path. Fine features such as neural fibers are shown in the zoomed images on the right. b) Z-stack of an E6 quail embryo prepared similarly. The Tuj1 stained neural fibered imaged with a 0.63× microscope objective (Olympus MVXPLAPO 0.63×/0.15). Almost the entire quail embryo body (>10×10×10 mm volume) can be imaged in a single Z-stack with good optical sectioning across the entire FOV. A zoomed image of the boxed region is shown on the right. c) Data in b displayed as a maximum intensity projection with the depth color encoded from 3 planes. XY represents the focal plane of the detection optics. Rendered videos of the datasets are shown in Visualizations Visualization 8 and Visualization 9.
the Z-direction (Fig. 7(a)). In addition, using a low magnification 0.63× macro objective with more than 11-mm FOV, the system is capable of acquiring an entire E6 quail embryo body in a single Z-stack (Fig. 7(b),(c)).

4.7. Resolution

The detection optics and the camera format determines the lateral resolution of the light-sheet microscope. Our primary setup uses a 0.5 NA 10× multi-immersion objective (Nikon CFI Plan Apo 10XC Glyc). By calculation, the diffraction-limited lateral resolution is below 0.6 µm, requiring a <0.3-µm pixel size for Nyquist sampling of the image. With the 2048×2048 sCMOS (Hamamatsu C11440-22CU) sampling in a 1.35-mm FOV, each pixel is around 0.65 µm. Therefore, the lateral resolution of the system is pixel-limited. Similarly, all of the lower magnification objectives used in this study have limited pixel resolution by calculation. The axial resolution is determined by the light-sheet thickness, which is essentially limited by the galvo mirrors’ clear aperture size (6 mm) and the illumination objective’s focal power (NA 0.28, 28.8-mm working distance). Without knowing the objective’s actual optical design, it is difficult to model the exact point spread function. Assuming the system’s aperture diameter is 6 mm, and the main lobe of the focused beam is close to a Gaussian profile, the approximate NA of the whole system is around 0.1. The corresponding beam waist radius is around 1.5 µm, and the estimated confocal range is about 120 µm.

To characterize the system’s practical resolution, we further measured the resolution using a fluorescent beads-phantom dispersed in a 1.5% agarose gel. The Gaussian-fitted FWHM axial resolution of 121 automatically detected beads is 3.89 ± 0.34 µm across the FOV. This represents a conservative average resolution of the system with errors associated with the non-uniform field (e.g., resolution variation across the FOV), quantization (e.g., limited sampling), and other noise. To verify the maximum resolution of the system, we also manually measured some selected beads. As shown in Fig. S10, diffraction-limited (200 nm) fluorescent beads only occupy 1 to 4 pixels in the lateral plane (some beads are near the center of 4 pixels). The beads’ FWHM axial resolution is ~5 pixels (0.65-µm isotropic sampling), corresponding to ~3.3 µm axial resolution. These measured resolutions are slightly worse than the calculated results (3 µm for NA = 0.1), potentially due to the presence of spherical and other aberrations resulted from the thick liquid interface between the sample and the objective lens. It is also worth noting that the system’s optical resolution is limited by light scattering and other optical aberrations induced by sample geometry Fig. S11. It is difficult to achieve the perfect optical resolution with semitransparent biological samples, especially deep into the tissue.

4.8. Optical sectioning

To compare the optical sectioning capability of the ASLM with the standard Gaussian beam configuration at different NA, we imaged the beam profile in a mildly scattering medium without galvo scanning. With ASLM, the FWHM of the ASLM beam profile is uniform (~4.4 µm) across the entire FOV at 10× magnification (Fig. S12). In contrast, a high-NA Gaussian beam (6-mm pupil diameter) with a thinner (~3.3 µm) sheet at the focus showed a highly diverging beam profile across the FOV. The low-NA Gaussian beam (2 mm pupil diameter) with a more uniform beam diameter across the FOV has a wider beam waist (~9-10 µm). This problem is more pronounced as the FOV increases, which is resolved with ASLM.

4.9. Beam uniformity

Telecentricity and flat field are two important factors affecting the uniformity of the scanned and swept light sheet. By setting the galvo in a fixed spot and enabling the ETL focus sweeping, we measured the angular deviation of the illuminated line to the optical axis of the illumination optics. We then changed the galvo position to test the angular deviation across the 5.8-mm FOV.
Angles of the beam vary from -0.1° to 0.6°, indicating a small non-telecentricity that can result in a 1.4% intensity difference across the FOV. The intensity difference is due to the line not being fully parallel to the pixels in the line-scan camera. The beam diameter is also uniform across the FOV. Considering more significant and unpredictable intensity variations due to scattering and absorption from the tissue, the minor intensity variation due to non-telecentricity is negligible in this application. To determine the field flatness of the illumination optics, we measured the location of the focus of 17 Gaussian beams across the entire FOV (Fig. S14). The automatically detected location of the focus varied by 15 pixels along the optical axis, corresponding to 10 µm. Considering that the Rayleigh length of the beam is greater than 100 µm, this variation ensures that the thinnest regions of the axially swept beams are synchronized to the rolling shutter across the FOV.

### 4.10. Alignment

Compared to a conventional customized light-sheet microscope, the CompassLSM is easier to align and maintain during assembly. The ETL is the only element in the design that requires a relatively precise alignment, achieved with a simple fold mirror pair. During operation, additional alignment is required to synchronize the swept light sheet with the rolling shutter, as discussed in section 2.8 and Fig. S3. In addition to synchronizing the axially swept light sheet to the rolling shutter, the exposure time and the galvo scan frequency must also be matched to ensure the light sheet uniformly covers the entire FOV (Fig. S15,16). Mismatched galvo frequency and exposure may result in image artifacts. Besides, it is desirable to use the lowest possible frequency to drive the galvo. The high frequency may result in reduced scan width and brighter edges (Fig. S17). It may also reduce the lifespan of the galvo.

### 4.11. Comparison to other ASLM designs

As discussed in Section 2.4, previous ASLM designs have a wide range of design specifications. The original ASLM by Dean et al. [12] is a high-resolution (<1 µm), high-magnification (>10×, < 1 mm FOV) design based on the remote focusing concept [31], which requires a highly complicated optical configuration (Fig. 1(f)) and a long optical path (>1 m). In contrast, CompassLSM has a significantly simpler configuration, smaller footprint, and shorter optical path length (<0.3 m). In addition, CompassLSM is designed to accommodate a larger FOV (<10×, up to 11 mm FOV). Although the optical sectioning is thicker (~3.5 µm) than the original design, the axial resolution is often sufficient for imaging cleared whole-mount tissue samples that are often millimeters in size.

The ASLM design by Hedde et al. [13] used much fewer optical elements than the remote focusing-based design [12]. Unfortunately, the alignment of the system is still difficult as it involves multiple 4f relays, including a pair of cylindrical lenses that requires a matched rotational alignment (Fig. 1(g)). It can still take significant effort to build, align, and maintain such a customized system. Besides, like the ASLM design by Dean et al., Hedde’s design has a thinner optical sectioning and smaller FOV compared to CompassLSM, less ideal for cleared tissue imaging.

The mesoSPIM design by Voigt et al. provides a larger FOV (up to 22 mm) and thicker optical sectioning (~6.5 µm) than CompassLSM (~3.5 µm). The thinner optical sectioning is beneficial for resolving large sub-cellular features such as cell nuclei. Besides, the optical and optomechanical configuration of mesoSPIM is more complicated because the off-the-shelf optomechanics introduce large degrees of freedom in the alignment procedure. In comparison, with convenient online fabrication services, customized optomechanics of CompassLSM drastically simplifies the design and the assembly procedure by lowering the total degrees of freedom in the system. The location tolerances of the optical elements (e.g., objective and ETL) are also significantly higher without relay optics.
4.12. Design constraints and potential modifications

The present CompassLSM design is optimized for cleared tissue imaging. There are tradeoffs between the imaging speed, resolution, and FOV. CompassLSM can be modified for different imaging applications (e.g., high-speed imaging) by changing the design parameters. Here, we review the key design constraints of the current CompassLSM and discuss potential strategies to overcome these limitations.

High-speed LSFM is useful for live sample imaging. The ultimate speed limit of CompassLSM is the camera’s frame rate (Hamamatsu C11440-22CU, up to 50 fps in the rolling shutter mode). When a faster imaging speed is desired, it is possible to surpass this limit using a different camera (e.g., a smaller sensor with 512×512 pixels). In our scanning setup, the imaging speed is further limited to <20 fps because the Z-stacking is achieved with discrete serial communication, which often takes tens of milliseconds for each Z-position to settle. A different stage control protocol (e.g., continuous analog control) can overcome this speed limitation. The ETL and the galvos can scan at >100 Hz resonant frequency. They do not limit the imaging speed in the current design, but they can become the limiting factor after other speed constraints are changed.

Some LSFM applications require a higher resolution. The NA of the detection objective lens primarily limits the lateral resolution of the system. A high NA, long working distance microscope objective is often costly. The axial resolution of CompassLSM is limited by the NA of the illumination, which is determined by the beam diameter and working distance (focal length) of the illumination objective. The beam diameter is currently limited by the aperture size of the galvo (6 mm). A larger galvo (up to 10 mm) can increase the NA of the illumination, but it will lead to a lower scan speed, limiting the overall time resolution of the system. Alternatively, using an objective lens with a shorter working distance can also increase the NA of the illumination and thus the resolution of the system, but it will reduce the back focal length of the objective and make it more difficult to physically fit a galvo.

The FOV is maximized in the CompassLSM design. A larger FOV can result in a more dispersed illumination, requiring a longer exposure time to maintain the SNR and limiting the imaging speed. The low-magnification XLFLUOR 4x/0.28NA objective with a long back focal length is also essential to achieve the large FOV demonstrated in this work. However, when such a large FOV is not needed, it is possible to use a different objective and trade FOV for higher resolution and imaging speed. In the present work, we have not identified an alternative lens option for a different FOV. The exact specifications of the proposed changes need to be empirically evaluated on a case-by-case basis for each new design and hardware choice because vendors usually do not disclose important specifications of the objective lenses, such as the back focal length.

5. Conclusion

We presented the design concept and the blueprint for CompassLSM, a compact ASLM light-sheet microscope that is easy to build, coding-free, and low-maintenance. We demonstrate the performance and example data generated by CompassLSM. Our design drastically simplifies the optical configuration of ASLM and provides uniform, micron-level optical sectioning across a multi-millimeter large FOV, larger than existing LSFM with similar optical sectioning capability. We believe this design is suitable for labs with some experience in simple light-sheet microscopy for large cleared-tissue imaging. With detection optics and a motorized stage already built, CompassLSM can be an upgrade to an existing Gaussian-based LSFM system. It is also a good option for space and budget-limited core facilities that handle a wide range of biological specimens. With a small footprint, there is also a potential to build CompassLSM into existing microscopes. The simple design concept and robustness can apply to other light-sheet microscope designs. It can also serve as a foundation for future open-source light-sheet microscope projects.
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Supplemental document. See Supplement 1 for supporting content.

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