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FULL ARTICLE

Integrative microendoscopic system combined with conventional microscope for live animal tissue imaging

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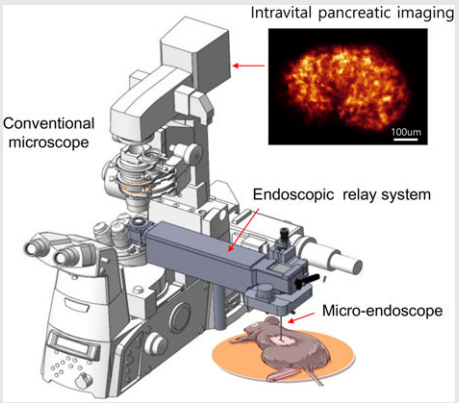
Intravital optical imaging technology is essential for minimally invasive optical diagnosis and treatment in small animal disease models. High-resolution imaging requires high-resolution optical probes, and high-resolution optical imaging systems based on highly precise and advanced technologies and therefore, associated with high-system costs. Besides, in order to acquire small animal live images, special types of animal imaging setups are indispensable. In this paper, a microendoscopic system is designed as an add-on to existing conventional imaging microscopes, reducing the price of complete confocal endomicroscopic systems. The proposed attachable system can be configured for confocal microscopes from common manufacturers and this enables users to acquire live animal cellular images from a conventional system. It features a 4f optical plane relay system, a rotary stage for side-view endoscopic probes, and an endoscopic probe mount which swings between the horizontal and the vertical. The system could be widely useful for biological studies of animal physiology and disease models.



KEYWORDS

confocal microscopy, endoscopes, intravital microscopy, optical devices

Martin Köhler and Bjorn Paulson contributed equally to this study.

1 | INTRODUCTION

Confocal microendoscopy aims to provide researchers with the technology for in situ optical diagnosis and treatment in small animal disease models [1–3]. By supplying micron-scale probes for microscopic imaging of living tissue [4–6], minimally invasive imaging procedure could be possible, while extending the potential working range of microscopy beyond the objective tip [6–10]. Recent advances in the miniaturization of small-diameter fiber bundles and GRIN (Gradient-index) lenses have enabled in vivo imaging of the mouse brain, kidney, and liver, as well as monitoring of tumor progression in murine colon models [5, 6, 11–18]. While less common in human applications, small-diameter confocal microendoscopic probes have been applied to visualize the bronchial tubes and airways in vivo [19–21], as well as in gynecology and pediatrics. By extending the spatial range of microscopy and moving large-diameter optical objectives away from the region of measurement, cellular-resolution endoscopic microprobes also promise to allow in-vivo optical biopsy of previously inaccessible tissues [9, 10, 21, 22].

As a result of recent technological developments, several robust design strategies for in vivo confocal endomicroscopy have emerged, and such systems are available from Optiscan, Karl Storz, LaVision Biotec and Mauna Kea Technologies, with probe diameters ranging from several millimeters to hundreds of microns for small animal use [23–26]. The Mauna Kea Mini probe, Karl Storz Coloview probes (300 μm diameter and 1.2 mm diameter, respectively) and the Optiscan confocal scanning probe (3.5 mm diameter) can only be attached to their own endoscopy systems. The LaVision panoramic view endomicroscope (2 mm diameter) is designed for microscopes from many manufacturers, but only allows for side-view imaging [27]. Of the commercially available systems, all include an integrated imaging unit which cannot be used for standard microscopy. Thus a system for the integration of micron-scale endoscopy probes with standalone optical microscopes is highly needed and would contribute greatly to low-cost and adaptable small animal research.

In this paper, we present a novel attachable module which mounts endoscopy probes of any diameter to freestanding confocal microscopes, expanding the capability of the microscope from in vitro to in vivo imaging of small animal models. For extended physical access to small animal models, the module extends the microscope's optical path 30 cm via a 4*f* optical relay system, and allows mounting of microendoscopic probes in horizontal and vertical orientations. For ease of use with any microscope, the module adapts to all standard microscope nosepieces, and features a rotating microscope junction. Preliminary visualization via micron-scale front- and side-view GRIN probes is demonstrated, and the system facilitates placement and manipulation of probes via both translation and rotation stages. In vivo fluorescence cellular imaging of colon and pancreatic cells in mice is demonstrated by this novel system, revealing the system's suitability for further biological studies of animal disease models.

2 | EXPERIMENTAL

The attachable microendoscopy module is combined with a confocal microscope and an endoscope to form a complete confocal microendoscopy system. The attachable module can be separated into three major components: the microscope junction, relay optics, and the swinging endoscope mount section. As depicted in Figure 1, light from the

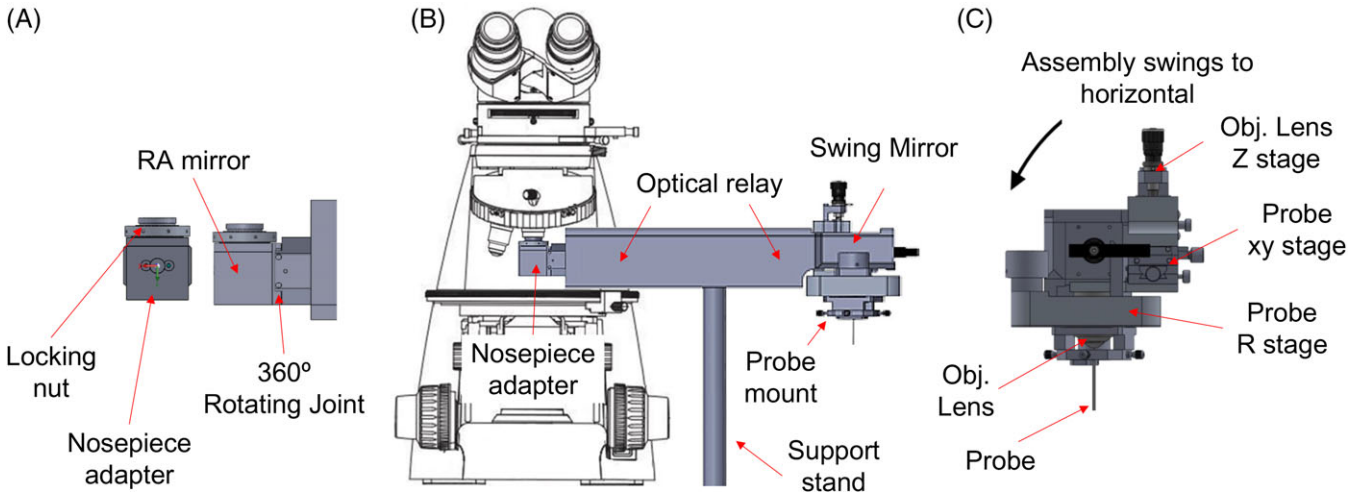


FIGURE 1 Schematic illustrations of the attachable microendoscopic system. (A) Front and side-views of the attachment junction, which consists of a nosepiece adapter on a rotating joint and contains a relay mirror. (B) The attachable module is shown attached to an upright microscope. Light is relayed from the nosepiece adapter to the probe mount. (C) Detailed side-view of the endoscope and objective mounting section. The entire section swings about the final relay mirror, which reflects light to the objective (Obj.) lens. The probe is adjustable in x-y, and may be rotated using the rotational stage (R stage), for performance of a full range of small animal experiments

microscope's optic path is launched into the endoscope via the microscope junction in lieu of an objective lens, which is depicted in Figure 1A. Beams on the optic path are reflected by a mirror in the junction and pass through a lens relay, which is fitted on to the microscope as depicted in Figure 1B. The horizontal beam is reflected into either the perpendicular horizontal or vertical by a swinging mirror, and the delivered light is focused on the endoscope probe by an objective lens in front of the endoscope probe mount, as shown in Figure 1C. Light reflected or emitted from the sample goes symmetrically back through the attachable microendoscopy system, allowing imaging of the sample by the microscope.

Microscope junction integrates with common confocal microscopes: The optical module needs to integrate seamlessly into a variety of existing conventional microscope systems. Consequently, the microscope junction is designed with a highly modular and configurable nosepiece adapter, which serves the dual purposes of stabilizing the optical connection between the microscope and the relay optics, and of minimizing stray light or dust entry into the imaging system of the microscope. As shown in Figure 1A, the nosepiece adapter body houses a protected silver relay mirror, and is flanked by a 360° rotating joint and a locking nut for the microscope junction.

Junction with conventional microscope via *universal thread:* The microscope junction is designed to be attached to the nosepiece of the microscope in lieu of an objective lens, and supports a universal thread adapter system which allows hassle-free connection between the endoscope system and any upright or inverted optical microscope with a mainstream optical microscope turret. It is shown in detail in Figure 2. A locking nut secures an externally-threaded C-mount adapter, which can also be attached directly to common CCD (Charge-coupled device) cameras. This adapter can be replaced with a standard externally RMS-threaded holder, which supports Olympus- and Nikon-brand nosepieces, and the RMS (Royal Microscopical Society)-threaded holder can be further extended by RMS to M25, RMS to M27, and RMS to M32 adapters for Nikon-, Zeiss-, and Leica-brand nosepieces. The nosepiece attachment schemas are detailed in Table 1.

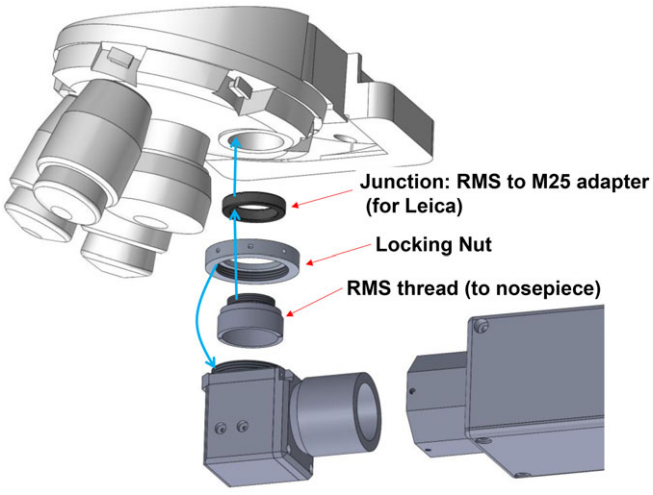


FIGURE 2 The adaptable junction attaches the microendsocope system to a variety of conventional microscopes, as shown in this schematic diagram. The full range of adapters shown in Table 1 is made possible by a design in which an RMS base is connected to the junction body by a locking nut (only holes shown)

TABLE 1 Table of microscope nosepiece threads by manufacturer

Nosepiece threads	Internally threaded
Zeiss	M27 or RMS
Leica	M25 or M32
Nikon	M25 or RMS
Olympus	RMS

The rotating joint is designed to so that the nosepiece adapter can be rotated around the horizontal axis, which enables connection to both upright and inverted microscopes, as well as to microscope nosepieces which are not perfectly horizontal or vertical. The rotating joint is held in place by between two and four M3 setscrews, which can be loosened and re-tightened after the junction has rotated freely around the horizontal beam axis. Combined with the adapters, the attachable module seamlessly connects confocal microscopes from major microscope manufacturers such as Leica, Zeiss, Nikon and Olympus.

The nosepiece adapter body transfers the image plane from the vertical microscope nosepiece to the horizontal 4*f* relay system (Figure 2) using a rectangular protected silver mirror with >90% reflection from 450 to 2000 nm. This visible-infrared mirror can be substituted with a cold mirror for heat-sensitive visible-light applications by replacement or disassembly of the nosepiece, and its angle can be fine-tuned within ±1° via two setscrews in the nosepiece adapter body.

4f relay system for light delivery: Practical application of confocal endomicroscopy requires that the coupling from the microscope's objective to the endoscope probe is not limited by the physical geometry of the confocal microscope. The attachable endoscopy module contains a 4*f* optical relay system which extends the physical range of the microscope beyond that which would be possible with direct coupling, and which transfers the image plane of the microscope to the probe, thereby extending the microscope to where it can easily be used for in vivo endoscopy experiments. The relay system is constructed as shown in the inset to Figure 3, and consists of two pairs of achromatic doublets, each with a focal length f = 200 mm (Edmund, NA = 0.07). At a separation of 2*f*, these lens pairs form a 1 to 1 nonmagnifying relay of the image plane. As the objective mount and objective lens are matched to an optical distance f from the lens pairs on each end of the system, the system does not require use of infinity-corrected objectives. A detailed schematic of

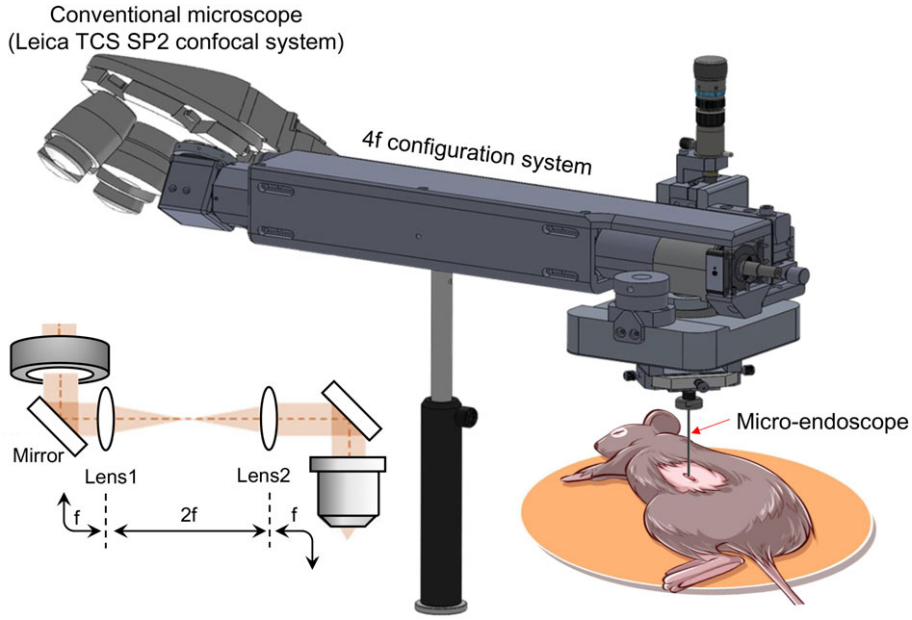


FIGURE 3 The 4F optical relay system frees the application of the microendoscope from the spatial constraints of the confocal microscopy imaging system. (Lower left) The optical relay consists of two lenses and two mirrors, which transfer the focal plane of the microscope to the probe-coupling objective lens. (Lower right) The mount also adds several spatial degrees of freedom while minimizing aberrations and optical losses. In this schematic, the attachable endoscopic system is applied for mouse pancreatic imaging

the optical path, including the 4*f* relay, mirrors, objective lens, and endoscope in the context of the device body is shown in Figure S1, Supporting Information. The system is also well-suited for beam scanning microscopes, because it minimizes transverse aberrations. To further reduce the aberration in this design, nearly plano-convex doublets can be used, and total light collection from fluorescence can be improved about 8% by using lenses coated for antireflection at the fluorescence emission wavelength. The final focal plane of the resulting image can be adjusted by axial translation of the endoscope-coupled objective lens or of the endoscopy probe mount.

The main body of Figure 3 shows the attachable microendoscopy module fixed to a commercial upright confocal microscope. A motorized translation stage is utilized to place and scan the sample animal laterally. The microendoscopy probe is inserted and fixed in a hole in the endoscope mount. The complete microendoscopy system combined with a confocal microscope is used for in vivo fluorescence imaging of internal organs in a mouse. As a result, the conventional microscope system is thereby expanded with the endoscopy modality for a much reduced cost as compared with a dedicated stand-alone endoscopy system.

Endoscope probe mount with rotation stage: For in-vivo imaging of small animal models such as mice, the required endoscopy probe mounting conditions vary with the measurement that is to be taken. A horizontal alignment is suitable for side viewing probe imaging of tubular organs such as the mouse esophagus and colon, while a vertical alignment is desirable for rigid-probe imaging of inner organs such as brain, kidney, and pancreas [15, 28]. Furthermore, it is desirable to be able to rotate and translate side-view probes with minimal jitter to create 360° cylindrical reconstructions of the target organ [5, 6]. These demands are accommodated by allowing the distal mirror, objective, and probe mount assembly to jointly rotate about the horizontal beam axis, so that the distal beam path may be moved between the vertical and the horizontal orientations as shown in Figure 4A-C. Vertical mounting of a rigid GRIN endoscope probe is shown in Figure 4B, while horizontal mounting with a rotational stage is shown in Figure 4C. The proximal probe tip is directly mounted in a v-groove based universal fiber screw-clamp, as shown in Figure 4B and Figure S2. For panoramic imaging with side-view endoscope probes, this probe mount is bolted to the distal end of a rotational stage (R-stage), as shown in the horizontal orientation in Figure 4C. Focus and imaging plane are controlled by an adjustable z-axis axial stage which translates the objective mount relative to the swing mirror and endoscope probe, and the R-stage on which the endoscope probe is mounted is translated by two single-axis transverse stages, which allow the center of the R-stage, and thus the probe center, to be moved in the transverse dimensions (Figure S3, Supporting Information). The objective is mounted independently of the R-stage via an internally-threaded C-mount thread which is set for transverse alignment during manufacture (Table 2).

The proximal microendoscope mount supports probes with diameters from 0.25 to 3.0 mm, and may be applied to the capture of 360° images with coupled side-view endoscopy probes, if supported by the rotational stage (R-stage), for the capture of cylindrical mosaic panorama images with coupled side-view endoscopy probes, as shown in

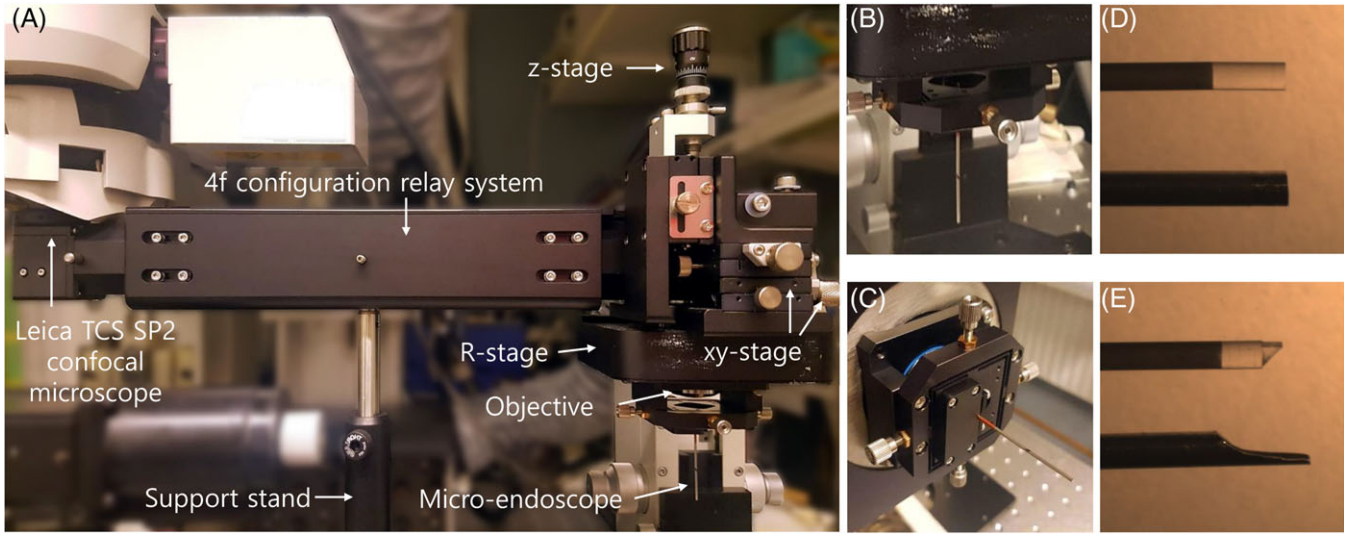


FIGURE 4 The fully assembled attachable microendoscopy system in application. (A) The relay system connecting a Leica TCS SP2 confocal microscope to a GRIN lens endoscope, with major parts for coupling and controlling the microendoscope probe marked. (R-stage: rotary stage) (B) a vertically orientated mount of the microendoscope probe allows imaging of mouse pancreas, liver, and kidney. (C) a horizontal mount of the probe allows for imaging of mouse colon, esophagous, and trachea. (D) Profile images of a front-view GRIN probe and protective sleeve which have been mounted in the attachable microendoscopy system. (upper: Front-view end tip, lower: protective sleeve) (E) Profile images of a side-view GRIN probe and protective needle which have been mounted on the attachable microendoscopy system. (upper: Side-view end tip, lower: protective sleeve)

Figure 4C. The manually-controlled R-stage is geared 8:1 for 45° of probe rotation per revolution, and allows rotational return accuracy to 0.45° per marked gradation, allowing for quick and accurate rotational scanning.

Front- and Side-view GRIN endoscope probes: While the endoscope system can mount endoscopy probe diameters ranging from 0.25 to 3.0 mm, probes in the range of 0.35 to 1.0 mm are most commonly used for minimally invasive imaging of live mice. For preliminary experiments, both front-view and side-view rigid GRIN probes were demonstrated, due to their nonpixelated image transfer and resultant higher transverse optical resolution in comparison to fiber bundle probes. It should be noted that other types of endoscopy probes, such as flexible fiber-bundles and flexible fiber-bundles with distal lens assemblies, can also be mounted on this system. Due to the desire for a simple imaging system compatible with standard microscopes, scanning endoscope probes are not supported.

Front-view and side-view triplet GRIN lens probes 1.0 mm in diameter and 62 mm in length were prepared following previously published methods [6]. The front-view triplet GRIN lens probe is shown in Figure 4D, and was used to image the mouse pancreas following the method described below. It is shown with a stainless steel protective sleeve of diameter 1.25 mm, which supports and protects the lens in vivo [6]. Front-view probes are appropriate for abdominal imaging of most visceral organs, such as the liver, spleen, or kidney [6, 17, 18, 28]. A side-view probe prepared by adhering an angled mirror to the distal GRIN lens facet is often more appropriate for imaging of the gastrointestinal and respiratory tracts such as the colon, esophagus or trachea, as well as for neural imaging [5, 6, 15, 16, 29–32]. The prepared side-view probe was fabricated using a 0.95 mm aluminum-coated prism, and is shown in Figure 4E along with a stainless steel protective sleeve having a diameter of 1.25 mm. Thus, the attachable endoscopy module supports a wide variety of probes. Based on experimental purposes and imaging requirements, the most suitable type, diameter and length of probe may be selected.

TABLE 2 Table of externally-threaded nosepiece mounts and internally-threaded adapters for the objective lens mounts

Probe-relay input thread (nosepiece side)	Externally threaded (nosepiece side)	Internally threaded (objective lens side)
Mount types	C-mount external	RMS internal
	RMS external	
Adapters	RMS to M25 external	RMS to M25 internal
	RMS to M27 internal	RMS to M27 internal

The prepared probes demonstrated transverse and axial resolutions of 1 and 11 μm in air, respectively, with a field of view of 195 μm for the front view probe and 230 μm for the side view probe, in agreement with previous results and theory [6]. In application, both GRIN lens triplet endoscope probes were coupled to the attachable endoscope mount in the same manner. The front-view probe demonstrated internal organ such as mouse pancreas and the side-view probe revealed tubular organ such as mouse colon.

Imaging setup and parameters: A Leica TCS SP2 confocal system was used with scanning laser illumination at 488 nm for excitation and imaging of the green fluorescent protein (GFP) and Fluorescein isothiocyanate (FITC). The attachable endoscopy system was coupled via the M25 nosepiece junction, and an infinity-corrected objective lens (NA 0.65, ×40, Olympus, NA 0.4, ×20, Zeiss, Oberkochen,

Germany) was focused on the proximal endoscope probe tip such that the image of the proximal end of the endoscope probe matched the dimensions of the camera sensor and illumination source. Excitation was performed at 488 nm and broad emission bands of FITC and MIP-GFP were detected [32]. For demonstration of the front- and side-view probe's scanning capabilities, mouse pancreas and colon images were stitched together in software following capture, according to previously published procedures [5]. Images were collected at a size of 512 by 512 pixels, and there is no need for special correction due to severe motion artifacts and due to contact imaging in the pancreas and in the mural colon.

Experimental mice: For demonstration of the optical setup, a mouse pancreas islet was imaged in front-view, vertical orientation, and a mouse colon model was imaged in side-view, horizontal orientation. The mouse is generally the most frequently used animal model for pre-clinical imaging [33], and the mouse colon is commonly used as a model to demonstrate tumors [25, 34, 35].

For pancreatic imaging, a transgenic mouse that expresses GFP under the control of the mouse insulin I gene promoter (MIP), 6 weeks old, was anesthetized according to standard protocols. The mouse was immobilized, and a small incision of 3 mm was made for inserting the endoscope into the pancreas. For colon imaging, a mouse, 6 weeks old, was intravenously administered with FITC 0.5 hours prior to examination. Both experiments were performed using general anesthesia (2.5% isoflurane in oxygen 0.4 L min^{-1}) with the mice immobilized on a motorized three-axis translation stage. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the ASAN Institute for Life Sciences, ASAN Medical Center, and the Animal Ethics Committee of Northern Stockholm, Sweden. The committee also followed the guidelines set by the Institute of Laboratory Animal Resources.

3 | RESULTS AND DISCUSSION

3.1 | Performance of the attachable endoscope module as an imaging platform

Lateral and axial resolution of the attachable microendoscopy system in air were measured with a front-facing GRIN lens probe of diameter 1.0 mm using USAF 1951 bar groups. The measured lateral and axial resolution of the attachable microendoscopy system are 1 μm and 11 μm, respectively (Movie S1, Supporting Information). This achieved resolution is sufficiently high for live cell imaging applications. The optical penetration depth of GRIN probes is limited to about 100 μm in most organ tissues. Sufficient image quality for video capture was obtained using the setup described with 30 frames per second. Due to the single fluorescent dye used, achromatism of the light path was not a large concern. Illumination wavelength and fluorescent wavelength were not very separated, and no achromatism was visible in testing. Modulation transfer function analysis of the attachable system without GRIN lens was performed based on the slanted edge method, and is shown in Figure S4. The MTF of the attachable system showed a modulation factor of 50% at 330 lp/mm, and reduction to 10% contrast at 800 lp/mm, which is comparable to standard confocal microscopy systems. A more detailed analysis of the optical contrast and resolution of the attachable system will be included in forthcoming publications.

3.2 | In vivo fluorescence imaging

Efficacy of the attachable endoscopic module for monitoring of disease development in mouse models was validated in horizontal and vertical GRIN probe orientation with side- and front-view probes, respectively. The Leica laser confocal microscope setup described in the Setup section was used with illumination at 488 nm and collection between 500 and 540 nm. A front-view GRIN triplet probe was used for fluorescence imaging of pancreatic islets in mice. A representative two-dimensional mosaic image from live transgenic MIP-GFP mouse pancreas with pancreatic islet beta-cells is shown in Figure 5. The image shows a texturing of individual beta cells, and is sufficient for histological characterization of pancreatic tumors or other disease models.

Using the same setup with a side-view GRIN probe, a diagnostic composite image was made of the mouse colon. The endoscope was first introduced toward the proximal end of the rectum, and images were taken continuously while extracting the endoscope backward. The resultant composite image, as shown in Figure 6, reveals vascularization of the colon lumen. Features such as capillary blood vessels of diameter less than 10 μm are clearly visible, showing the

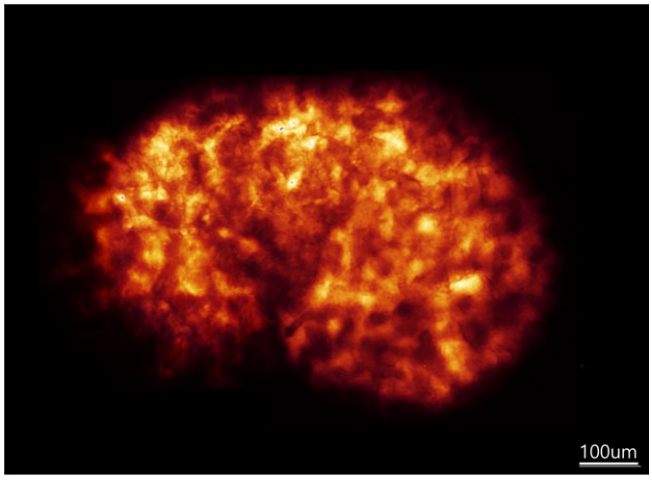


FIGURE 5 In vivo pancreas endoscope mosaic imaging of MIP-GFP mouse shows cellular resolution using a rigid front-view GRIN probe in vertical mount orientation. The 1.0 mm diameter probe was connected via the attachable endoscope system to a Leica TCS SP2 confocal system for imaging. Excitation was performed at 488 nm

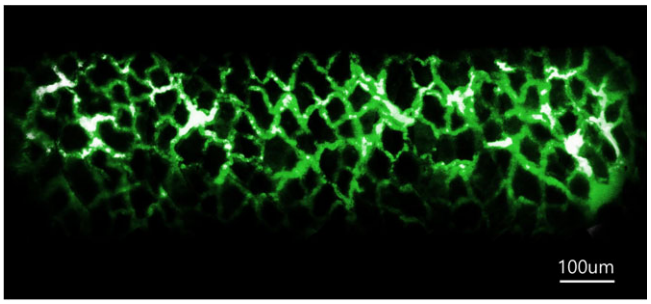


FIGURE 6 In vivo colon fluorescence imaging using the attachable microendoscopy system and a rigid side-view GRIN microendoscopy probe shows mouse colon vasculature at cellular resolution. Staining was performed with FITC dye (green). The 1.0 mm diameter probe was mounted in horizontal orientation, and the composite image was stitched together using a series of images with 488 nm excitation

potential for cellular tissue histology. The images attained are suitable for tumor propagation studies in vivo.

4 | CONCLUSION

This study demonstrates the feasibility of an attachable module that adds a microendoscope with confocal endoscopic capabilities to a standard confocal microscope. Preliminary experiments clearly demonstrate the ability of the system to perform in vivo imaging, including single-cell imaging, tissue histology, and tumor progression studies in small-animal models. Microendoscopic probes in both side- and front-view allow for in vivo, in situ cellular-resolution morphological and functional imaging from a standard upright confocal microscope. The total manufacturing cost of the device was approximately USD 6000. This device is of particular utility for low-cost small animal fluorescence endoscopy, where access to small internal organs is essential, and will be particularly useful for laboratories with standalone microscopes which would like to extend their existing technology to endoscopic imaging modalities. For human clinical use, the use of a stand-alone microscope is less practical. However, the attachable microendoscopy system is ideal for the fast development and testing of small-diameter microendoscopy probes, as will be required for the next generation of minimally invasive medical diagnosis. In conclusion, the development of the attachable microendoscopic system, with its modular design and practical imaging modalities, paves the way to low-cost confocal microendoscopic imaging modalities in small animal disease models.

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Please see Supporting Information online.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Detailed cutaway of the device design, with a focus on the optical components. Components shown in blue are mirror mounts, and in black are lens mounts, which each immobilize two achromatic doublets in the optical path. The objective lens in green is held in the beam path and connected to the Z-stage by the microscope mount, shown in gold.

Figure S2 Engineering schematic of the probe mount, showing a semitransparent probe mounted in the v-groove, and immobilized by the screw clamp.

Figure S3 Engineering schematic of the objective mount, showing its connection to the z-stage and independence from the rotational stage. The entire objective mount and probe assembly swings with the swing mirror.

Figure S4 Modulation transfer function (MTF) analysis of the attachable relay system. (A) Image of a USAF 1951 target. (B) MTF as calculated from the slanted line method.

Movie S1 Z-stack movie of the FITC injected murine vasculature (30um depth with 3um steps)

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