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Invited Paper

Optical fibers for high-resolution *in vivo* microendoscopic fluorescence imaging

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ABSTRACT

Optical fiber-based high-resolution fluorescence imaging techniques have promising applications in clinical practice and preclinical research using animals. Here we review the instrumentation and applications of microendoscopy based on various types of optical fibers. Single-mode fibers and double-clad fibers have been widely used for delivering light from light sources to tissues and collecting light from tissues to photodetectors. Coherent fiber bundles, cylindrical graded-index lenses, and multi-mode fibers have been employed in both beam-scanning and non-scanning microscopy. With continuing advances of optical fiber technologies, further innovations in optical microendoscopy are expected.

1. Introduction

Over the past decades endoscopes have been used to observe inside of hollow cavity or the surface of inner organs of the human body for diagnosis or surgery in a minimally invasive or noninvasive manner [1]. An endoscope can transmit light deep into the body through optical fibers and visualize lesions that are typically inaccessible by other means. However, it has several limitations in detecting small lesions or identifying underlying microscopic pathological features. For example, the missing rate of colonoscopic diagnosis can be as high as 22% to detect adenoma [2]. Regular colonoscopy procedures cannot provide histopathologic information by itself, and sometimes require unnecessary biopsy or removal of suspected lesions for diagnosis, which often requires patients to return for additional biopsies or examinations.

Nowadays, optical fiber technology has advanced to perform real time assessment of tissue pathology. High-resolution endoscopy can help improving diagnosis of a microscopic lesions or help delineating tumor margins more accurately. This instrument is referred to as a "microendoscope" or "endomicroscope" (see Ref. [3]). Together with the development of high-resolution endoscope, fluorescence imaging has increased applications to the medical field. As fluorescence probes can be designed to bind specific target molecules, a potential indicator of pathology, fluorescence imaging allows endoscopists to identify suspicious lesions with altered fluorescence as potential biopsy. Fluorescence microendoscopy can provide distinct contrast arising from characteristics of lesions in addition to reflected white light [4].

In this review, we describe state-of-the-art fiber-based fluorescence imaging modalities using various types of optical fibers, emerging imaging techniques, and promising medical applications where microendoscopy can improve the current clinical practice.

2. The types of optical fiber for microendoscopic fluorescence imaging

The optical fiber is a key element for implementing microendoscopy for flexible maneuvering along curved lumen of animal or human organ, which is usually impractical with rigid elements. Various microendosocpic imaging modalities based on the type of optical fibers are illustrated with their specific applications.

2.1. Fiber bundles

Fiber bundles commonly consist of ~100,000 individual step-index fibers (the total diameter of fiber bundle: hundreds of micrometers to a few millimeters, core diameter: 2.0–4.0 μm, core-to-core distance: 3.2–6.0 μm). Each higher-refractive index core is embedded in a common lower-refractive index cladding that act as a single pixel of an image. They allow transmission of intensity in pixelated form and spatial arrangements of each fiber

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at both ends are identical with coherent fiber bundle. Wide-field endoscopic imaging can be performed with fiber bundle without the need of scanning. On the other hand, proximal scanning confocal imaging is also used with fiber bundle as each core serves as a confocal pinhole [5,6].

The use of fluorescent dyes for microendoscopy based on fiber bundle improves the sensitivity of detection [7,8]. Typical fluorescence microendoscope system is composed of a light source, a fiber bundle, and a combination of lenses and a CCD camera. The excitation light sources are lasers, LEDs or lamps. After the excitation light exits the distal end of the fiber bundle to illuminate sample, the emitted fluorescence is collected by the fiber probe and is sent through an objective, a dichroic mirror, and an emission filter prior to forming an image onto a CCD camera (Fig. 1(a)). Fiber bundles have been used in various imaging modalities including confocal microscope which is originally invented by Marvin Minisky in 1955 [9]. General confocal microscope is operated based on point light illumination. Detection of light signal through a pinhole from a corresponding specimen point allows the rejection of out-of-focus background. This confocal operation can provide high-resolution three-dimensional volume imaging by scanning the focal spot laterally and axially. Raster scanning is ordinarily performed in the transverse plane perpendicular to the optical axis at fixed depth with respect to the tissue surface [10]. Confocal or other scanning microendoscopes employ either proximal or distal scanning. Proximal (meaning *near from the point of attachment*) and distal (meaning *away from the point of attachment*) scanning is based on the location of scanner from an endoscopist's point of view. In proximal scanning, the imaging probe does not contain scanner and there is no restriction on the size of scanner. In contrast, distal scanning requires developing miniature endoscopic probe that contains scanning unit. In proximal scanning, individual fiber within a fiber bundle is used as the pinhole while scanning is performed across the fiber bundle. The advantage of this method is the unlimited size of scanning system as it is not included in the microendoscopic probe. Gmitro et al. demonstrated fluorescence confocal imaging using a fiber bundle by raster scanning [11]. Proximal scanning systems for confocal imaging use one or more moving mirrors to scan the confocal pinholes across the fiber bundle [12]. Ovarian cancer screening has been attempted in the peritoneal cavity using confocal imaging modality based on fiber bundle [13].

Confocal fluorescence microendoscopy has been applied to the medical area. Cellvizio (Mauna Kea Technologies, Paris, France), a commercial system for *in vivo* fluorescence microendoscopic imaging, has shown real-time microscopic images of urinary tract, gastrointestinal and respiratory epithelium (Fig. 2) [16–20]. High-resolution confocal imaging probe based on fiber bundle and attached GRIN lens has been also developed to produce sub-micron resolution imaging of tissue samples [14,15].

However, there are several disadvantages of confocal microendoscopy. Even with its high spatial resolution, the penetration depth is typically less than 100 μm from tissue surface [21]. Furthermore, confocal imaging suffers from excessive photobleaching limiting its capability of time-lapse imaging. These limitations can be overcome by two-photon fluorescence imaging [22]. Two-photon excitation using a fiber bundle allows flexible, small endoscopic probe capable of deep imaging in tissue. Göbel et al. presented a miniature two-photon microscope based on a fiber bundle with a gradient-index (GRIN) lens, and imaged fluorescently labeled blood vessels in rat brain *in vivo* (Fig. 1(b)) [15].

The main disadvantage of conventional fiber bundles is the pixelation artifact due to the non-imaging spaces between fiber cores and core-to-core distance between fibers. As a result, fiber bundles have limited lateral resolution in the sample plane. Furthermore imaging contrast degrades due to the optical crosstalk through the layer of cladding between adjacent fiber cores [6]. To reduce pixelation artifact, various image processing methods have been developed. Obtaining multiple images and performing computational post-processing can improve the limited resolution of fiber bundle [23]. Kyrish et al. used a custom electromechanical actuator in order to acquire the shifting pattern of the specimen: Multiple shifting patterns were assessed to increase lateral resolution by combining and realigning the shifted images using imaging algorithms. Han et al. presented another method to remove the pixelation artifact of the fiber bundle structure by applying combined histogram equalization and by post-processing images with a Gaussian spatial smoothing filter [24]. Other group proposed an algorithm to remove artifacts by finding particular peaks in Fourier domain based on morphological *processing* and notch filter [25].

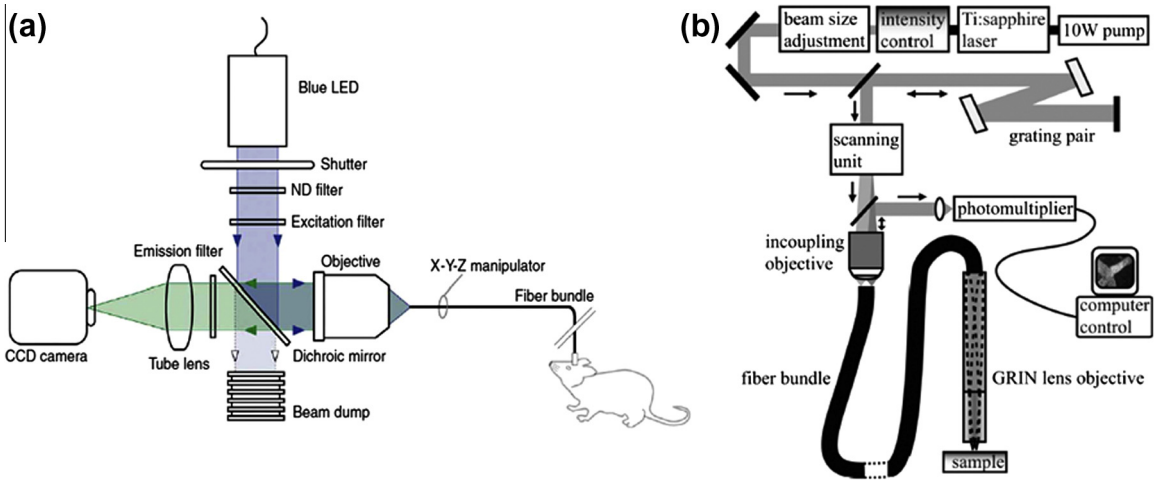

Fig. 1. Schematics of fluorescence microendoscope with fiber bundle. (a) One-photon modality, an epifluorescence microscope attached to fiber optic system for *in vivo* imaging. (From Murayama, M. and M.E. Larkum, *In vivo dendritic calcium imaging with a fiberoptic periscope system*. Nature protocols, 2009. 4(10): p. 1551–1559; with permission) [89]. (b) Two-photon modality, Femtosecond laser pulses pass a pair of diffraction gratings for pulse compression before they are coupled into fiber bundle through a standard two-photon laser-scanning microscope. Fluorescent emission is detected through the fiber bundle and the in coupling objective by a photomultiplier tube. (From Göbel, W., et al., *Miniaturized two-photon microscope based on a flexible coherent fiber bundle and a gradient-index lens objective.* Optics letters, 2004. 29(21): p. 2521–2523; with permission) [15].

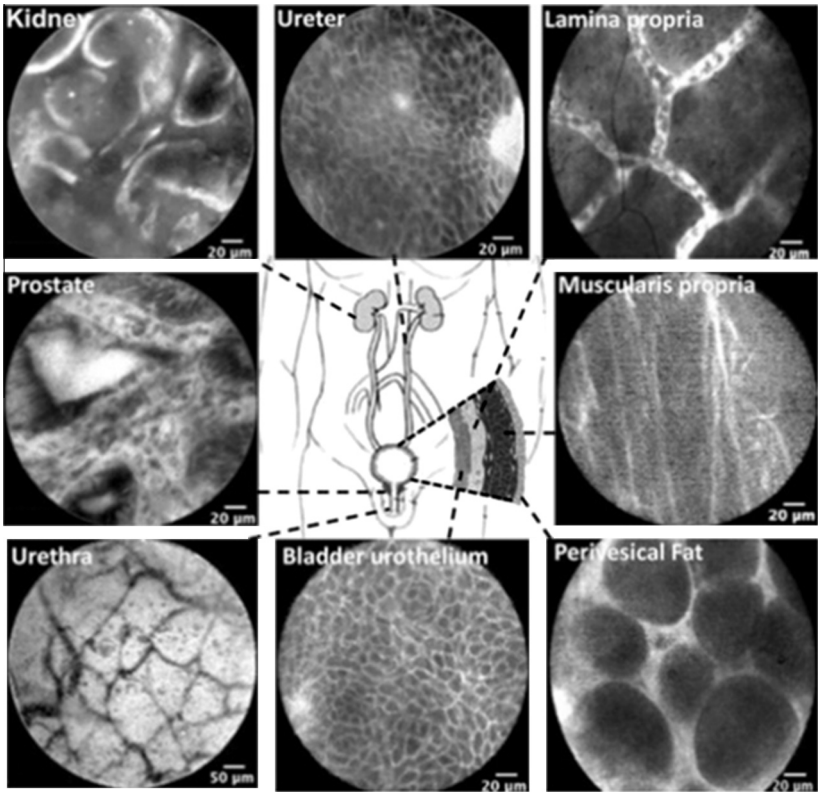

Fig. 2. Confocal microendoscopic images of the normal urinary tract using the Cellvizio system. All images of the lower urinary tract were acquired *in vivo*, whereas upper tract images (kidney cortex and ureter) were acquired ex vivo. Note similarities of the urothelium (intermediate cells) between ureter and bladder. Lamina propria is characterized by capillary network of moving erythrocytes. Muscularis propia and perivesical fat images were obtained from the tumor resection bed. (From Wu, K., et al., *Dynamic real-time microscopy of the urinary tract using confocal laser endomicroscopy*. Urology, 2011. 78(1): p. 225–231; with permission) [20].

2.2. Single-mode fiber

Single-mode fiber has a very small core diameter of only a few microns (<10 μm). As a subclass of step-index fibers, single-mode fiber has only a single spatial mode of light. Step-index fibers, known as conventional fibers, have two layers with different refractive indices. The inner core surrounded by outer cladding can transmit light by total internal reflection [26]. The single-mode fiber is well suited for the scanning-based imaging modalities as the single spatial mode can be focused to a near diffraction-limited spot in the specimen plane for high-resolution imaging. In order to obtain 2D or 3D images, a miniaturized scanning mechanism is required inside the imaging head. While proximal scanning with a fiber bundle gives limited optical resolution due to the spacing between individual fibers, distal scanning with single-mode fiber does not suffer from this problem by bringing scanning unit to a miniature endoscopic probe (Fig. 3). Some rigid endoscopes utilize a flexible single-mode fiber as confocal pinhole and employ relatively large scanning systems at the distal end of an optical fiber (Fig. 4). Two-dimensional mechanical scanning of a lensed fiber as an input into a long GRIN relay lens has also been shown to reduce off-axis aberrations.

The single-mode fiber core that delivers light can also act as a pinhole detector by rejecting out-of-focal plane fluorescence emissions [27]. Giniunas et al. built and tested a scanning confocal microscope using a single-mode optical fiber for illumination and collection [28]. Two main forms of distal scanning are currently in use or under development. One uses a miniature mirror or mirrors to scan the pinholes across the tissue to be imaged. A second method involves a combination of moving the coupling fiber only in one direction and using wavelength-specific optical diffraction to create a line of pinholes, each of which conducts only a specific wavelength range of light to and from the targeted sample.

Tearney et al. introduced the concept of spectrally encoded confocal microscopy [29]. A broadband source is dispersed laterally by a diffraction grating so that the spatial position is encoded in the wavelength. All wavelengths can be transmitted through a single fiber and the spatial position of the reflected light can be decoded by spectral detection [30]. A dual prism grating element was used to miniaturize the probe head for spectrally encoded imaging modality [31]

The imaging modality has been emerged as a non-scanning technique using single-mode fiber to overcome the pixelation artifact of optical fiber bundle imaging. The spectrally encoded endoscopy (SEE) enables video-rate, three-dimensional images with probe diameter comparable to that of a human hair (Fig. 5(a)) [33]. SEE uses miniature diffractive optics like a grating to encode space information into spectrum, and can provide the number of pixels larger than that of fiber bundle depending on the spectral encoding capability [34]. Through a single optical fiber, polychromatic light irradiates the tissue within the body. The reflected light can be decoded outside of the body by using a spectrometer that forms one line of endoscopic image. By moving the fiber using a mechanical-transduction mechanism, SEE provides two-dimensional image. Furthermore, spectral encoding can provide depth information by using optical interferometry [35]. As a demonstration, SEE imaged metastatic ovarian tumor nodules on the partial peritoneal wall *in vivo* (Fig. 5(b)). Though fluorescence SEE imaging is realizable, it has to overcome several limitations: high back reflections from the probe, inefficient fluorescence encoding, and pronounced speckle noise [34,36]. Lately, Abramov et al. reported how to go around these issues by multiple-channel spectrally en-

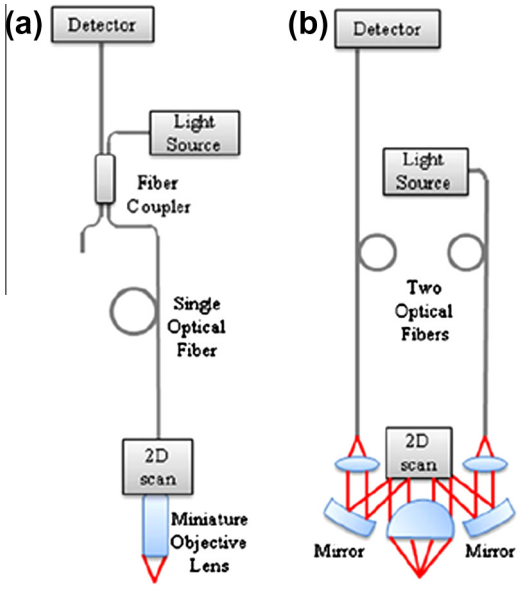

Fig. 3. Confocal microendoscope architectures. (a) Distally scanned microendoscope using a single optical fiber for illumination and detection. Scanning can be accomplished by mechanically moving a fiber, by a fiber with a miniature lens, by a MEMS mirror, or by spectrally encoding one axis and mechanically scanning the second axis. (b) Distally scanned microendoscope using two optical fibers, one for illumination and the other for detection, in a dual-axes confocal configuration with a MEMS scanning mirror. (From Jabbour, J.M., et al., *Confocal Endomicroscopy: Instrumentation and Medical Applications.* Annals of biomedical engineering, 2012: p. 1–20; with permission) [3].

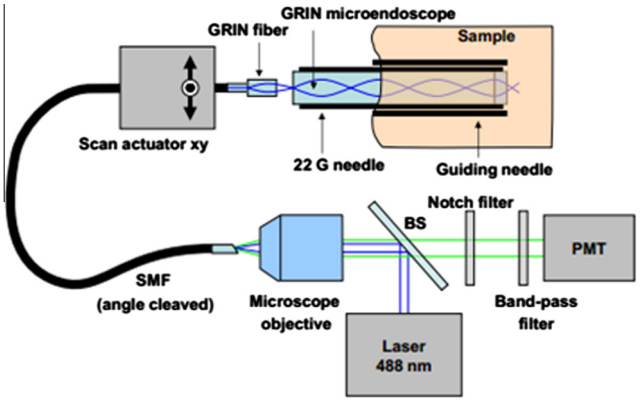

Fig. 4. The schematic of confocal microendoscope based on single-mode fiber. BS: dichroic beamsplitter, PMT photomultiplier tube. (From Pillai, R.S., D. Lorenser, and D.D. Sampson, *Deep-tissue access with confocal fluorescence microendoscopy through hypodermic needles.* Optics Express, 2011. 19(8): p. 7213–7221; with permission) [32].

coded endoscopy (MC-SEE) [34]. In MC-SEE the spectrally encoded imaging and illumination channels are separated to produce high sensitivity fluorescence imaging. By using a high numerical aperture imaging lens to improve signal collection efficiency, they showed fluorescence image of single cells (Fig. 5(c)) [34].

Another type of microendoscopes employs electromagnetic or piezoelectric actuators to distally scan the fiber and objective lens together. In 1994, Dickensheets and Kino presented the use of a resonant cantilever to scan a single fiber with a Fresnel zone plate miniature objective lens [37]. Later, microendoscope with resonant single optical fiber has been developed by the Seibel group [6].

Endoscopes based on optical fiber bundle suffer from poor image quality by the limited number of pixels and honeycomb-like image artifact produced by non-imaging space between optical cores [6]. A novel imaging modality, the scanning fiber endoscopy (SFE) has emerged to resolve these issues by actively scanning an amplitude-modulated resonating fiber [6,38,39]. The SFE system consists of a single-mode optical fiber, piezoelectric actuator tube to generate vibratory resonance, scanner housing, and a detector to record the time-multiplexed backscattered signal. SFE can also generate color image with red, green and blue laser source coupled into a single fiber. The piezoelectric actuator tube drives the fiber tip at its resonant frequency in an expanding pattern of 350 spirals, generating two-dimensional scan patterns at a frame rate of 15 Hz. While the projected light is focused onto the sample plane by the lens assembly, the backscattered or emitted light is collected through collection fibers to separate the collection pathway from the illumination pathway. The image is generated by computer image processing and can be displayed in real time (Fig. 6). With high-resolution and wide field of view, SFE has been tested in large lumens of organs of human upper digestive tract such as esophagus and stomach [6]. Notably, SFE resolution is limited by the lens assembly and not by the number of detector pixels as in camera or optical fiber bundle. Recently a multispectral scanning fiber endoscope was demonstrated to collect *in vivo* wide-field fluorescence images from multiple peptides binding specifically to colonic dysplasia [5]. The SFE technology, with its pixel-level control of laser sources, can be used for therapeutic applications in addition to imaging.

2.3. Double-clad fiber

Double-clad fiber (DCF) consists of triple layers of optical material. The inner-most layer serves as core. The core is surrounded by the inner and outer claddings. The three layers are made of materials with different refractive indices. There are two different kinds of double-clad fibers. The first was developed early in optical fiber history with the purpose of engineering the dispersion of optical fibers. In these fibers, the core carries the majority of the light, and the inner and outer cladding, alter waveguide dispersion of the core-guided signal. The second type of the fiber was developed for use with high power fiber amplifiers and fiber lasers. The inner cladding and core together guide the pump light, which provides the energy needed to allow amplification in the core. These fibers are unique in their ability to support single-mode propagation through the core and multi-mode propagation through the inner cladding. DCF for two-photon imaging is fundamentally two fibers in one, and can accomplish both excitation pulse delivery and collection of emission fluorescence light. In spectrally encoded imaging, double-clad fiber was used to reduced speckle noise [36]. Yelin group demonstrated the use of a DCF for single-mode illumination and multi-mode detection to achieve high-resolution with a large depth of field. DCF can be used to enhance several other fiber-based imaging and non-imaging systems. It was used in multi-photon endoscopy that is desirable where light can be delivered through an optical fiber and images can be acquired using a miniature imaging probe (Fig. 7). Other groups used DCF to deliver femtosecond pulses through the single-mode core, and multi-photon signal were collected through the multi-mode inner cladding [40,41]. Other multi-photon endoscope utilized a two-axis electrostatic MEMS scanner and DCF [42]. Rivera et al. presented a compact and flexible two-photon and second-harmonic generation endoscope that utilized a miniaturized resonant/nonresonant fiber raster scanner. The scanner was fabricated by mounting a DCF and multi-element GRIN lens assembly [43]. DCFs have been applied to confocal imaging [36]. Lemire-Renaud et al. presented a method for using DCF in confocal microendoscopy for preserving the optical sectioning. The device allows higher signal, reduces laser speckle noise with less significant loss in axial and lateral resolution compared to single-mode fiber based confocal microscopes [44]. Bao et al. developed a fast handheld two-photon fiber-optic

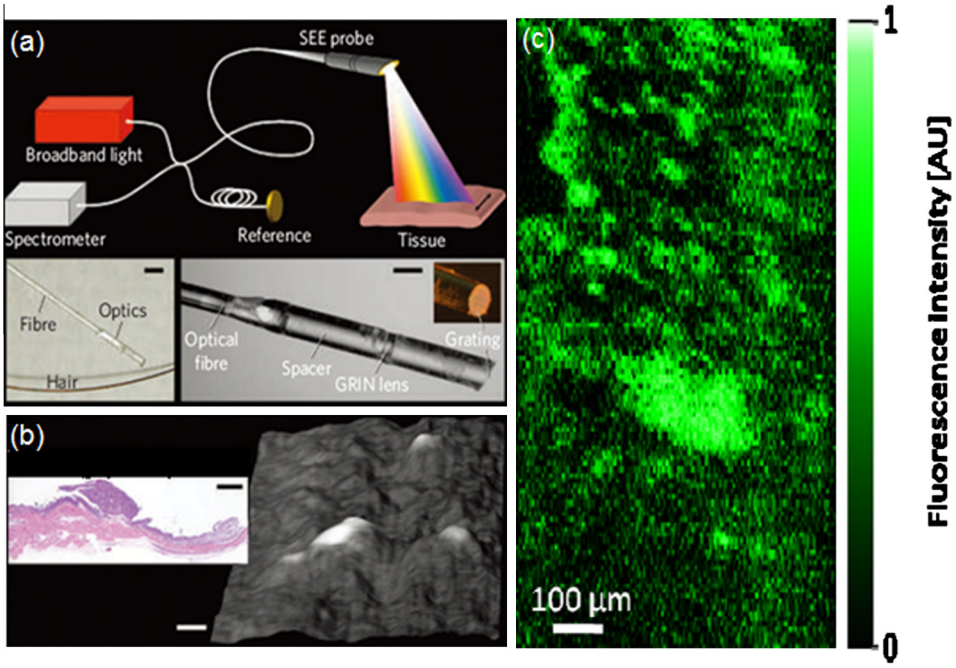

Fig. 5. Miniature, three-dimensional endoscopy through a single optical fiber. (a) The spectrally encoded endoscopy (SEE) system and probe; tissue is scanned orthogonally to the wavelength axis by rotating the probe.(b) Image of mouse ovarian tumor nodules on the parietal peritoneal wall *in vivo*. The reflectance image, shown in gray shades (bright grays indicate high reflectance), is superimposed on a three-dimensional rendering of the tissue surface from the corresponding area. Scale bars, 0.5 mm. (From Yelin, D., et al., *Three-dimensional miniature endoscopy*. Nature, 2006. 443(7113): p. 765–765; with permission) [33]. (c) Fluorescence imaging of cell culture using MC-SEE. (From Abramov, A., L. Minai, and D. Yelin, *Multiple-channel spectrally encoded imaging*. Optics Express, 2010. 18(14): p. 14745–14751; with permission) [34].

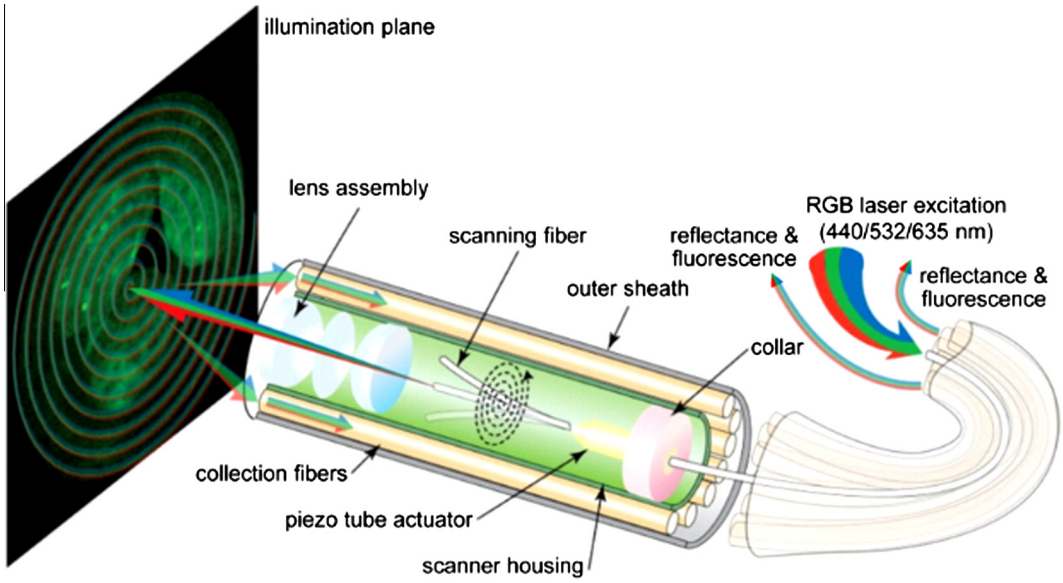

Fig. 6. Functional diagram of the Multispectral SFE with the scanning illumination fiber moving in the spiral scan pattern. RGB laser excitation (440, 532 and 635 nm) is delivered into a single-mode fiber that is scanned in a spiral pattern by a piezo tube actuator and focused onto the tissue (illumination plane) by a lens assembly. Fluorescence is collected by a ring of 12 collection fibers mounted around the periphery of the scanner housing, protected by an outer sheath. (From Miller, S.J., et al., *Targeted detection of murine colonic dysplasia in vivo with flexible multispectral scanning fiber endoscopy*. Journal of biomedical optics, 2012. 17(2): p. 021103-1–021103-11; with permisson) [90].

fluorescence endoscope for 3D *in vivo* cellular imaging. In this system, DCF was used for excitation light from Ti: sapphire laser (single-mode fiber) and collection from fluorescence signal (multi-mode fiber). Rivera et al. fabricated flexible multifocal two-photon microendoscope based on DCF to acquire SNR images at a 4 frames/s per plane without excessive tissue photodamage [45].

Novel multimodal microendoscopic imaging using DCF with optical frequency domain imaging (OFDI) and near-infrared fluorescence (NIRF) imaging has emerged to shed light on human coronary artery disease [47]. While OFDI provides three-dimensional images of arterial wall with higher frame rate [48], NIRF provides molecular information in arterial disease, such as inflammation in proteolytically active atherosclerosis [49]. These modalities are combined through a dual-modality rotary junction that rotates and pulls back the imaging probe that is composed of DCF and a ball lens. The imaging probe transmits light of OFDI and NIRF through separate concentric light-guiding channels of the double-clad fiber and focuses the beams into the sample. OFDI

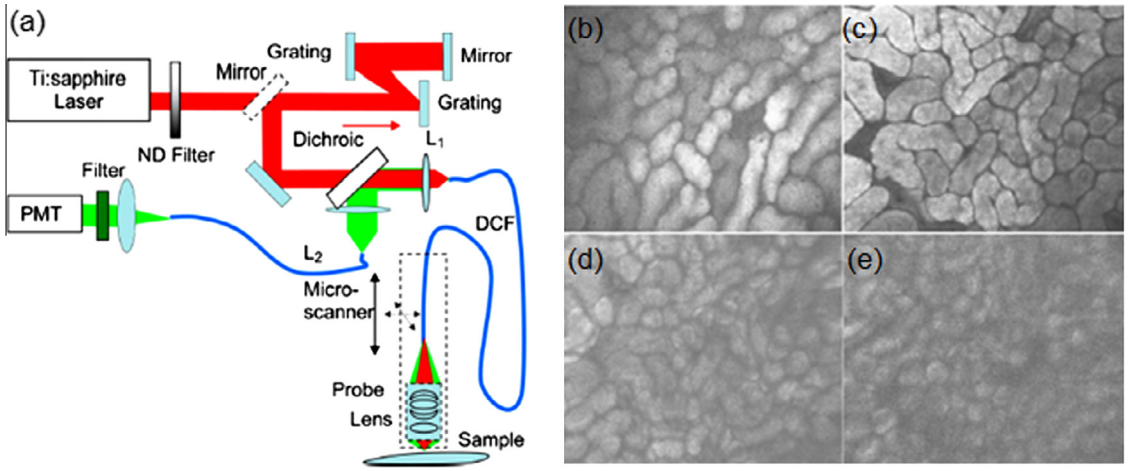

Fig. 7. Microendoscope based on double-clad fiber. (a) Schematic of two photon fluorescence endoscopic system. (b)–(e) Two photon fluorescence images of a rat kidney surface. 0, 20, 40, 60 μm deep from the surface. (From Bao, H., et al., *Fast handheld two-photon fluorescence microendoscope with a* 475 μm × 475 μm *field of view for in vivo imaging.* Optics letters, 2008. 33(12): p. 1333–1335; with permission) [46].

measures depth profile from spectral interference signal that is generated by swept-wavelength interferometer. Three-dimensional microstructural OFDI data is acquired by helical pullback scanning of the imaging probe. Simultaneously, NIRF signal that reports functional information is acquired at every depth profile acquisition (Fig. 8). In an animal model of atherosclerosis, morphological features as well as cysteine protease activities of atherosclerotic plaque were simultaneously measured by the combinational system *in vivo*. By simultaneously providing atherosclerotic plaque microstructure and molecular mechanisms of vascular pathophysiology, this novel approach could provide new opportunities for identifying high-risk plaques *in vivo* [50].

2.4. Multi-mode fiber

Multi-mode fibers with parallel information transport have more than one spatial mode of transmitting light. Their modes are analogous to the different standing wave patterns that form on the surface of the fiber [5]. There are two types of multi-mode fibers: one is step-index multi-mode fiber, with two layers of different refractive indices, and the other is gradient refractive index (GRIN) fiber that the index in core declines almost quadractically with radius from the central axis The former fibers are better suited for fluorescence collection than single-mode fiber due to larger core diameters, commonly ~50 μm up to a few millimeters, and greater numerical aperture values in general. The latter GRIN fiber has reduced modal dispersion due to the nearly parabolic common refractive index profile and continual refocusing of the rays in the core. Multi-mode fiber is suitable for shrinking the size of endoscopes based on fiber, because of having large number of degree of freedom. However, until recently multi-mode fibers have not been used for imaging purpose due to the wave distortion induced by the mode dispersion [51,52]. The solution to reconstruct a real image from the scrambled image has emerged by the theory of light propagation in a distorted medium [53]. Lately, an innovative endoscopy using a single multi-mode fiber for wide-field imaging has emerged by Choi's group [53]. Lensless microendoscopy by a single fiber (LMSF) is based on the existence of numerous independent spatial modes within a multi-mode fiber for parallel information transport. The group measured the transmission matrix, the input–output response of an arbitrary optical system by connecting the free modes at the input plane to those at the output plane, of a multi-mode optical fiber and converted it into a self-contained 3D imaging device without requiring a lens or attached scanner. The speckle pattern from the multiple and random reflections of light, being deterministic, can recover a clean image with a diffraction-limited resolution (Fig. 9). While fundamentally different from other single fiber microendoscopy techniques, LMSF has potentially advantages in image acquisition speed, pixel resolution and diameter of imaging unit. Furthermore, this LMSF can potentially be extended the fluorescence imaging.

Another imaging modalities based on multi-mode fiber have been developed to put back the scattering properties and to scan light through them using digital phase conjugation technique for generating a sharp focus at the end of the fiber or complex optimization algorithms using spatial light modulator [55,56]. To compensate the scattering of turbid media, Papadopoulos et al. demonstrated a microendoscope used digital phase conjugation technique to overcome the modal scrambling. Papadopoulos et al. demonstrated a microendoscope used digital phase conjugation technique to overcome the modal scrambling. They showed high quality fluorescence images of neuronal cells. One group presented a novel approach by utilizing disordered light within a standard step-index multi-mode optical fiber for lensless microscopy and optical mode conversion [57]. They demonstrated the modality of bright and dark-field imaging and scanning fluorescence microscopy with fast acquisition rates that allowed observation of dynamic processes such as Brownian motion of mesoscopic particles. Lately, Papadopoulos group demonstrated high-resolution fluorescence imaging using multi-mode fibers in conjunction with digital phase conjugation. They have achieved sub-micron resolution fluorescence imaging of fluorescently stained neuronal cells [58].

2.5. Graded index (GRIN) microlens

Non-scanning approach employs the GRIN microlens – similar in principle to the graded-index multi-mode fiber – as an imaging probe. GRIN lens with diameter of 350–1000 μm has cylindrical shape with radially-varying refractive index, and thus allow narrow diameter and simple mounting to rod lenses, the connecting components. GRIN lens has a special refractive index profile of $n(r) = n_0(1 - Ar^2/2)$, where n_0 is the refractive index at the center, $A^{1/2}$ is the gradient constant and r is the distance from the central axis. Unlike a conventional lens using curved surfaces to refract light, a GRIN lens uses the radial refractive index profile of a nearly parabolic shape to guide light with a cosine ray trace (Fig. 10) [6,14]. In a ray description, total internal reflection occurs in a

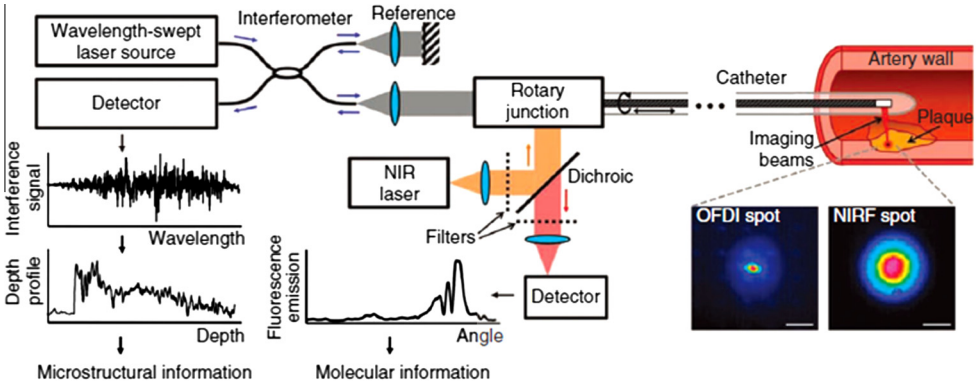

Fig. 8. Schematic of the dual-modality intra-arterial catheter for simultaneous microstructural and molecular imaging. In the OFDI system, the light source changes its wavelength rapidly as a function of time. The depth profile is reconstructed by taking the Fourier transform of the spectral interference signal that is generated when the reference and back-reflected signals from tissue microstructures are combined and detected. Depth profiles are continuously acquired to form cross-sectional images while the probe is rotating. NIR fluorescence emission, which reports molecular information, is also simultaneously acquired at depth profile acquisition. Scales bar is 100 μm. (From Yoo, H., et al., *Intra-arterial catheter for simultaneous microstructural and molecular imaging in vivo.* Nature medicine, 2011. 17(12): p. 1680–1684; with permission) [47].

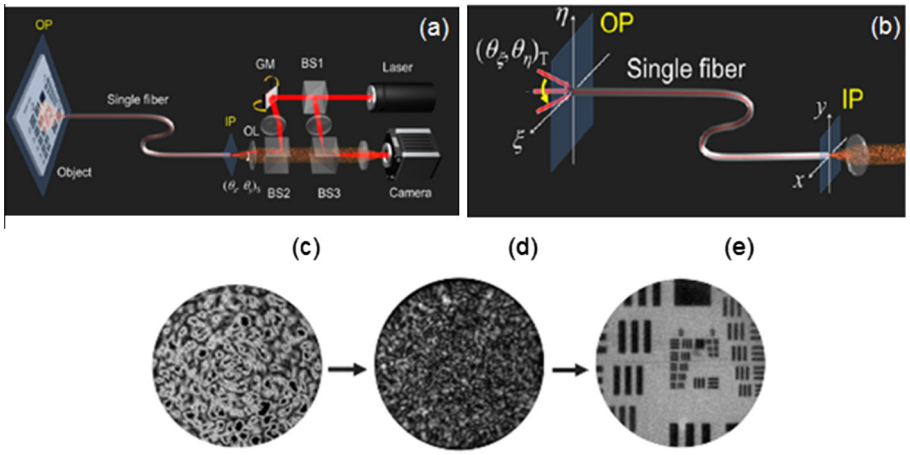

Fig. 9. New imaging modality using LMSF system. (a) Experimental scheme of LMSF. BS1, BS2 and BS3: beam splitters. OL: objective lens. IP: input plane of a multi-mode optical fiber. OP: object plane. (b) Scheme of a separate setup from for measuring a transmission matrix of the single fiber from OP to IP. The incident angle $(\theta_\xi, \theta_\eta)_T$ is scanned and the transmitted images are recorded. (c–e) Image reconstruction process. (c) Representative images of a measured transmission matrix following the scheme shown in (a). (d) Reconstructed object images after applying TLI (Turbid lens imaging) method. This turbid lens imaging is a method that converts a turbid medium into a lens [54]. (e) Averaging of all reconstructed images in (d). (From Choi, Y., et al., *Scanner-free and wide-field endoscopic imaging by using a single multi-mode optical fiber.* Physical review letters, 2012. 109(20): p. 203901; with permission) [53].

gradual manner as light passes from central regions to peripheral regions of lower refractive index, causing light rays to travel down the cylindrical lens axis in an approximately sinusoidal path [38].

This lens has been applied as noninvasive microendoscopic probe that can transfer high-resolution images with working distance of many centimeters [38,59–61]. Using GRIN microlens probe implanted in a mammalian brain, long-term and repetitive observation of pituitary gland has been demonstrated with the optical resolution of 2.5 μm [62], and fluorescently labeled neuron was visualized [38]. Recently, time-lapse cellular imaging of tumor growth within live mammalian brain has been shown using high-resolution microendoscope [63].

Several rigid confocal microendoscopes without optical fibers have been developed primarily for preclinical applications, these rigid microendoscopy designs typically have a small diameter lens system that can be inserted into an animal for intravital imaging. Scanning is performed proximal to the miniature lens system and the scanning system does not need to be miniaturized because it is usually located outside of the body [64]. Using a series of rod lenses with a 0.9 NA miniature objective lens, Farahati et al. developed a rigid confocal microendoscope in 2010 [65]. In the same year, Feng et al. showed the design of the scan lens and the objective lens arranged into one long tube for a confocal microendoscope [66]. Kim et al. describe a new approach based on a side-view micro endoscope with a prism at the distal end of the probe that enables wide-area cellular-level fluorescence imaging of tissue in live mice. Contact between the view window and luminal wall makes it easy to navigate along the tract by rotation and translation of the probe. This allowed for obtaining a comprehensive map of fluorescently labeled cells and microvasculature in the mu-

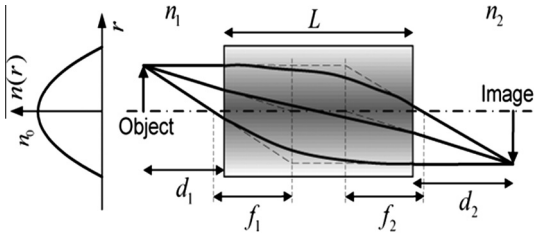

Fig. 10. Refractive index profile and image formation of a GRIN lens. (From Fu, L. and M. Gu, *Fibre-optic nonlinear optical microscopy and endoscopy.* Journal of Microscopy, 2007. 226(3): p. 195–206; with permission) [14].

cosa of various mouse organs *in vivo* at multiple time points (Fig. 12(c)) [64]. They presented longitudinal imaging of immune cells in renal allografts and tumor development in the colon [67].

Deep tissue fluorescence imaging in living animals has been expedited by multi-photon imaging approaches (Fig. 11). Two-photon laser scanning microscopy was originally invented by Watt Webb group in 1990 [69]. High photon density enabled by spatial focusing and temporal pulsing can generate non-linear optical process in which two excitation photons are simultaneously absorbed by a fluorophore. Two-photon excitation only occurs at the focal point and, by point-by-point scanning, this allows three-dimensional volumetric imaging. Consequently, no pinhole is necessary in front of detector and more photons scattered from fluorophore can be collected through the tissue. In addition to the absence of pinhole, the use of near-infrared of infrared excitation light enables deep tissue imaging since longer wavelength light undergoes less scattering and absorption than shorter wavelength light in typical tissues. GRIN microlens can be inserted in a live mammalian brain to visualize cells and dendritic spines for two-photon microendoscopy (Fig. 12(a)). Furthermore, it has been used to visualize time-lapse imaging of three-dimensional vasculature structure for observing tumor growth in the brain *in vivo* [63]. The Webb group showed that multi-photon microscopy through GRIN lenses enabled minimally invasive imaging with subcellular resolution, from several millimeters away from a lesion in an anesthetized animal. They presented *in vivo* images of cortical layer V and hippocampus in the anesthetized *Thy1-YFP* mouse [59]. Lately, the Schnitzer group used a microendoscope with custom-developed GRIN lens to track thousands of CA1 pyramidal cell's place fields over weeks in freely behaving mice [70].

Several fluorescence optical imaging modalities exploit these benefits of optical fibers and produce images using a few common fiber arrangements. All five categories of optical fiber can involve the high-resolution fluorescence imaging modalities reviewed elsewhere in this issue. Many advantages and limitations of each fiber are summarized in Table 1. We focus here on the role of optical fibers and imaging modalities that are to introduce various researches on microendoscopic fluorescence imaging to readers (Table 1).

3. Medical applications of microendoscopy

Early and accurate diagnosis is important for effective prevention and treatment of disease. Diagnosis of disease is often accomplished via evaluation of tissue biopsy samples. As confocal or two-photon endoscopy can achieve optical sectioning with high-resolution, these imaging modalities can be applied to the clinic. Two commercial confocal microendoscopy systems are currently in use in the clinic. The Pentax ISC-1000 (Pentax, Tokyo, Japan) confocal microendoscopy system is a miniaturized confocal endomicroscope that can be inserted into a conventional video-endoscope. A distally scanned single optical fiber serves as both the illumination and detection confocal pinhole. The beam is raster-scanned by scanning the fiber with an electromagnetically driven scanning mechanism [74]. The system was used to diagnose heterotopic gastric mucosa [75]. The Cellvizio is a standard imaging system based on a fiber bundle can be passed down the accessory channel of any standard endoscope, allowing for rapid image capture *in vivo* in real time. Unlike the distal scanning of Pentax system, the Cellvizio utilizes a proximally scanned fiber bundle to deliver excitation laser light to the sample. [1]. This system provides *in vivo* imaging of benign and neoplastic mucosa along the urinary tract [20].

In general optical endoscopy has been developed to provide noninvasive disease diagnosis particularly in cancer detection [7,76]. The morphological and molecular changes observed by microendoscopy can help clinicians monitor the progression of neoplastic disease [4]. Although white light has been adopted in the clinic to identify suspicious lesions for biopsy, the method can only provide limited information demanding higher resolution technology with sub-cellular resolution [7]. With the advancements presented in this review, microendoscoic probes may soon slim down their size sufficiently small to minimize the requirement of anesthesia and reduce tissue damage. Fluorescence enhances the diagnostic value of microendoscopy by revealing molecular information of target tissue [76]. In addition to non-specific contrast agents, targeted molecular specific contrast agents have recently been developed for potential use *in vivo* to target biomarkers associated with cancer [77]. For example, selected peptide using phage display technology that is screened for molecular detection was shown a promising potential for colorectal cancer diagnosis by the Wang group (Fig. 13) [76,78]. The combination of targeted probes and confocal microendoscope showed high sensitivity and specificity for detecting dysplastic colonocytes *in vivo* (Fig. 13. (c)).

The group performed first-in-human clinical study using confocal microendoscopy *in vivo* in 25 human subjects after topical peptide administration for detecting esophageal neoplasia. This experiment results show that this safe molecular imaging agent and fluorescence confocal microscopy based on optical fiber may

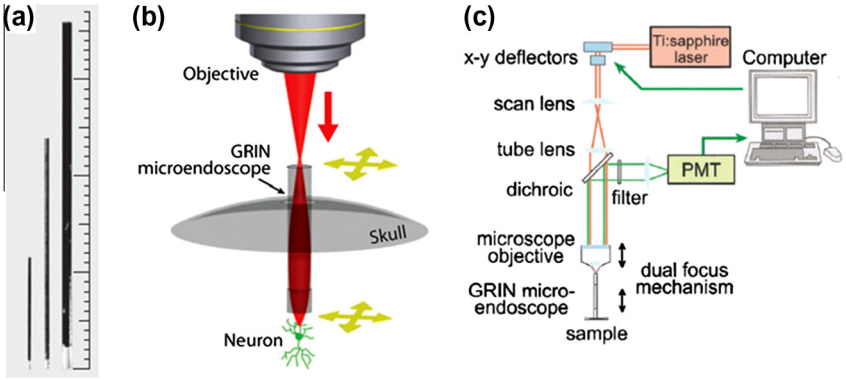

Fig. 11. *In vivo* optical microendoscopy based on GRIN lens. (a) Photograph of compound doublet microendoscope probes 350 μm, 500 μm, and 1000 μm in diameter. (b) Schematic of a microscope objective lens coupling illumination. (From Wilt, B.A., et al., *Advances in light microscopy for neuroscience.* Annual review of neuroscience, 2009. 32: p. 435; with permission) [68]. (c) Schematic of an optical microendoscope based on GRIN lens. The focal plane in the sample may be adjusted by movement of either the endoscope itself or the microscope objective that couples the excitation beam. (From Jung, J.C. and M.J. Schnitzer, *Multiphoton endoscopy.* Optics letters, 2003. 28(11): p. 902–904; with permission) [60].

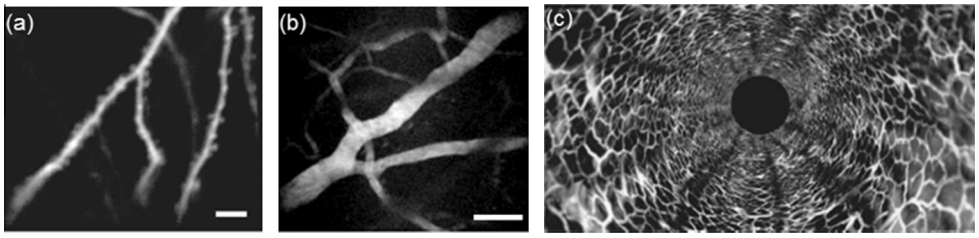

Fig. 12. *in vivo* fluorescence imaging based on GRIN lens. (a) Imaging with high-resolution micro-objectives permits superior resolution than = uncorrected GRIN lenses, and enables visualization of neuronal dendritic spines. Scale bar is 10 μm. (From Barretto, R.P., B. Messerschmidt, and M.J. Schnitzer, *In vivo fluorescence imaging with high-resolution microlenses.* Nature Methods, 2009. 6(7): p. 511–512; with permission) [71]. (b) Two-photon fluorescence image of blood vessels with free-flowing rhodamine-B-dextran conjugates after tail-vein injection (2,000,000 MW, 200 μg/200 μl) in an ear skin. Scale bar is 50 μm. (From Kim, P., et al., *In vivo confocal and multiphoton microendoscopy.* Journal of biomedical optics, 2008. 13(1): p. 010501–010501-3; with permission) [72]. (c) A fly-through rendered fluorescence image of the vasculature in the descending colon of a normal C57B6/L mouse. The wall-to-wall diameter is 1.3 mm. (From Kim, P., et al., *In vivo wide-area cellular imaging by side-view endomicroscopy.* Nature Methods, 2010. 7(4): p. 303–305; with permission) [64].

Table 1
The type of optical fibers for microendoscopic probe.

The type of optical fiber	Advantages	Limitations
1. Fiber bundle [6,23–25,32]	• Ease of use for imaging • For proximal scanning. No restriction of scanner size	• Pixelation artifact reduces effective resolution • Reduced image contrast due to optical crosstalk among individual fibers
2. Single-mode fiber [6,28,44]	• High-resolution imaging achievable with point scanning • Highly flexible with narrow diameter	• Require a scanning system at proximal or distal end • Sensitive to misalignment
3. Double-clad fiber [6,40,41]	• Separated excitation and fluorescence collection channel • Can deliver ultrashort optical pulse	• Require dispersion compensation for pulse excitation • Less flexible than single-mode fiber
4. Multi-mode fiber [6,53,56,57,73]	• Various imaging modes (i.e. bright and dark-field, and fluorescence) • Depth information can be retrieved from holographic imaging	• Direct imaging is not available due to wave distortion from multiple modes • Require post procession for image reconstruction
5. Graded index (GRIN) microlens [6,62–64,66]	• Allows high-resolution imaging • Various imaging modalities can be used (One-, Two-photon, Confocal)	• Rigid probe does not allow flexible maneuvering • Presence of optical aberration

be useful for guiding tissue biopsy and for early detection of esophageal neoplasia and potentially other cancers of epithelial origin, such as colon and lung [79].

The use of microendoscopy for diagnosis of cervical cancer has been explored by the Richards–Kortum group [7]. To conduct a pilot study for cervical cancer screening, they have developed a high-resolution fluorescence microendoscopy system based on fiber bundle (Fig. 14). The fluorescence dye, proflavine, labels DNA, and distinguishes nuclei from cytoplasm of the cell. As a result, important parameters such as nuclear size and nuclear-to-cytoplasmic ratio can be studied as they are histopathologic features of cervical cancer [80]. These developments are expected to find its use in the clinic for diagnosis of epithelial malignancies.

Two-photon imaging modality has become a novel optical tool of choice for imaging deep tissue architecture and cellular morphology. There are a number of researches using two-photon microscopy in medical fields to diagnosis for lung cancer, gastric cancer, esophageal cancer and bladder cancer [81–86]. The images alone provided sufficient detail to classify most lesions as either benign or neoplastic using the same basic diagnostic criteria as histopathology. Two-photon microscopy has yet to be used as clinical endoscopy. However the advancement of miniaturized two-photon imaging probe based on special optical fibers may enable a real-time *in vivo* diagnosis of early stage cancers at the cellular level. Webb group showed the application of two-photon microendoscopy for *in vivo* evaluation of lung cancer using mouse

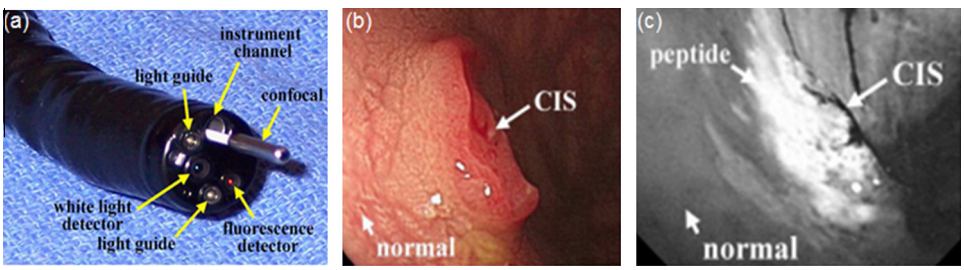

Fig. 13. Application of confocal microendoscope. (a) Confocal imaging instruments. (b and c) Targeted confocal imaging. (b) A dysplastic polyp sits on a colonic fold consisting of normal mucosa. (c) Confocal image following administration of fluorescent-labeled peptides demonstrates preferential binding to dysplastic crypt (left) comparison to adjacent normal crypt (right), scale bar 20 μm. (From Wang, T.D., *Targeted imaging of flat and depressed colonic neoplasms.* Gastrointestinal endoscopy clinics of North America, 2010. 20(3): p. 579; with permission) [78].

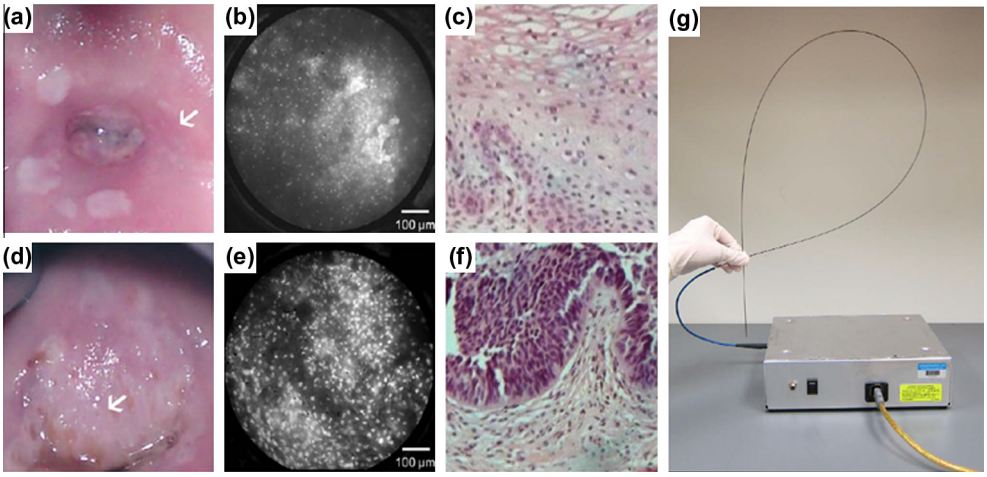

Fig. 14. Application of one-photon fibered high-resolution microendoscope (HRME) for diagnosis. The top row shows images obtained from a clinically normal region of the cervix. The white arrow in the colposcopic image (a) indicates the area imaged with the HRME. The HRME image (b) shows small, uniformly spaced nuclei, and was considered non-neoplastic by both subjective expert observers, which is consistent with histopathology indicating HPV effect (c). Histopathological imaging. The bottom row shows images from a clinically abnormal region of the cervix. The white arrow in the colposcopic image (d) indicates a region with a clinical impression of high grade disease. The corresponding HRME image (e) shows large, pleomorphic, crowded nuclei and was considered neoplastic by both subjective expert observers, which is consistent with histopathology indicating CIN3 (f). (g) Photograph of the high-resolution microendoscope (HRME). (From Quinn, M.K., et al., *High-Resolution Microendoscopy for the Detection of Cervical Neoplasia in Low-Resource Settings.* PLOS ONE, 2012. 7(9): p. e44924; with permission) [7].

models [87]. These studies will provide the groundwork for further use of two-photon microendoscopy in the clinic as a noninvasive "optical biopsy" tool.

4. Summary and perspective

Modern microscopic techniques using various optical fibers for high-resolution with fluorescence contrast have brought more clear view of suspicious lesion to physicians for better diagnosis and proper management. Microendoscopy based on various optical fibers has expanded the range of accessible tissues deep inside the body and resolutions down to cellular level in living experimental animals or humans in clinic. The design of endoscopic probes can be customized to accommodate a wide range of imaging conditions at significantly reduced cost for low-resource setting [7]. High-resolution optical imaging can provide histologic and pathologic information by evaluating cellular morphology and additional molecular probes. To compare various fluorescence microendoscopic imaging modalities, we categorized imaging modalities into non-scanning vs. scanning approach in terms of resolution, advantages/limitations. In addition we also included several emerging new imaging technologies (see Table 2).

There remain barriers for these microendosocpic technologies to be used in routine clinical use as confirmative gold standard such as histopathologic examination. To improve the utility of microendoscopy in the clinic, combination of complementary

Table 2
Comparison of fluorescence microendoscopic modalities.

Imaging modalities	Resolution (lateral/axial)	Advantages	Limitations
Non-scanning approach			
• One-photon microendoscopy [6–8,21,62]	• ~4 μm (fiber bundle probe) (lateral)	• Full-frame acquisition	• Lack of optical sectioning
	• ~2.5 μm (GRIN lens probe) (lateral)	• Ease of use	• Pixelation artifact reduces effective resolution
		• Low cost	
Scanning approaches			
• Confocal microendosocpy [6,10,19–22,64]	• (1–5) μm/(15–25) μm (lateral/axial)	• Micron resolution with pinhole aperture	• Penetration depth of <50 μm in tissue
		• Optical sectioning	• Relatively small field of view
		• Cellular resolution	• Excessive photobleaching
• Two-photon microendoscopy [6,21,22,38,45,71,88]	• ~0.8 μm/~10 μm (lateral/axial)	• Deeper penetration (<100 μm)	• High cost due to pulsed laser source
		• Reduced photobleaching and photodamage	• Relatively small field of view
		• Cellular resolution	
Emerging approaches			
• OFDI/NIRF multimodal endoscopy [50]	• ~30 μm/~7 μm (lateral/axial)	• Simultaneous microstructural and molecular imaging	• Complexity of system
• Scanning fiber endoscopy [6,39]	• 1 μm/~8 μm (lateral/axial)	• Ultrathin size of probe and wide-field of view	• Nonuniform sampling
		• Easy combine with other imaging modalities	• Nonideal dynamic behavior of actuator
• Spectrally encoded endoscopy [34–36]	• 145 μm/~7 μm (lateral/axial)	• Video rate, three-dimensional image, miniature probe available	• Inefficient signal collection requires bigger aperture lens
• Lensless microendoscopy by a single fiber [53]	• 1.8 μm/~13 μm (lateral/axial)	• Diffraction-limited resolution without aberration, and fast imaging acquisition potential	• Applicable only for partially flexible endoscopic operation

imaging modalities might be required. As existing technologies mature and novel technologies emerge, fluorescence-based micro-endoscopy using optical fibers will become a routine procedure to provide high-resolution, non-invasive 'optical biopsy' and on-site point diagnosis in the foreseeable future.

Acknowledgments

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