Advances of endomicroscopy for gastrointestinal physiology and diseases

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Goetz M, Kiesslich R. Advances of endomicroscopy for gastrointestinal physiology and diseases. Am J Physiol Gastrointest Liver Physiol 298: G797–G806, 2010. First published February 25, 2010; doi:10.1152/ajpgi.00027.2010.—Confocal endomicroscopy is a novel technique that permits in vivo microscopy of the human gastrointestinal mucosa during ongoing endoscopy, thereby providing optical virtual biopsies. Endomicroscopy has been demonstrated to reveal histological information in a multitude of diseases in the upper and lower gastrointestinal tract in vivo. Most studies have focused on inflammation and neoplasia, such as Barrett’s esophagus, gastric cancer, celiac disease, Crohn’s disease and ulcerative colitis, or colorectal neoplasias. Endomicroscopy allows obtaining of “smart,” targeted biopsies from regions with microscopic alterations rather than having to rely on random untargeted tissue sampling. This reduces the number of biopsies while increasing the diagnostic yield. In addition, immediate histological information is available, enabling immediate therapy. Apart from morphological visualization, endomicroscopy offers a unique possibility to study pathophysiological events in their natural environment (functional imaging). Molecular imaging with endomicroscopy applied in clinical and basic science will permit advances in understanding of the cellular basis of gastrointestinal physiology and pathology.

molecular imaging; Barrett’s esophagus; gastric cancer; celiac disease; ulcerative colitis; colorectal cancer

CONFOCAL ENDOMICROSCOPY has raised increasing interest since its first publication in 2004 (20). In contrast to chromoendoscopy or virtual image enhancement techniques, which try to predict histopathology, endomicroscopy is able to actually see histological details during ongoing endoscopy. Earlier techniques aiming for microscopic visualization in humans had incurred significant compromises as to resolution or applicability of bulky bench-top devices in clinical science and routine. With the miniaturization of scanning devices or fiber-bundle techniques, novel confocal devices have been developed that can be used during routine endoscopy. Compared with white-light microscopy, confocal laser microscopy allows high-resolution subsurface imaging in intact tissue by combining focal laser illumination with a pinhole to geometrically eliminate out-of-focus light.

Indications in which confocal endomicroscopy is evaluated are rapidly growing. All these studies share the aim to enhance diagnostic accuracy for on-table diagnosis during endoscopy. First trials indicate that endomicroscopic imaging results in taking fewer targeted (“smart”) biopsies instead of multiple random samples. It should be pointed out that endomicroscopy is a technique that should be used in a targeted fashion. It is ideally combined with a wide-field endoscopy technique such as chromoendoscopy, high-definition scopes, or virtual chromoendoscopy techniques that narrow the area of interest, which is then examined by endomicroscopy.

Just like any other advanced endoscopy technique, endomicroscopy needs training in the handling of the endoscope and interpretation of the images. Close collaboration with an expert histopathologist is recommended. The learning curve usually follows a two-step scheme in which the first step includes the differentiation of normal vs. abnormal mucosa on the basis of a simple-to-use classification system before a second step where detailed and subtle appreciation of microscopic detail is sought. It has been estimated for Barrett’s esophagus that 100 examinations are required after a supervised introduction to reliably perform endomicroscopy (5). Endomicroscopy is a unique tool to obtain in vivo histology and also offers an unprecedented opportunity to observe dynamic mucosal events throughout the gastrointestinal tract without major sampling, fixation, cutting, or staining artifacts.

Technique of Endomicroscopy and Staining Protocols

Technique of confocal endomicroscopy. The first human trials of endomicroscopy used a miniaturized scanner integrated into the distal tip of a conventional endoscope (EC-3870CIFK; Pentax, Tokyo, Japan). The scanner delivers an excitation wavelength of 488 nm at a maximum laser power of 1 mW via a single optical fiber. The imaging plane depth is adjustable from surface to 250 μm in depth, in 4-μm increments. Thus, similar to a CT scan, serial optical sections of 475 × 475 μm with a lateral resolution of 0.7 μm are obtained oriented parallel to the tissue surface (Fig. 1). The same scanner is integrated into a handheld probe for animal research (FIVE1; Optiscan, Notting Hill, Australia).
More recently, flexible confocal probes were introduced that can be passed through the working channel of most conventional endoscopes even into the bile duct (Cellvizio; Mauna Kea Technology, Paris, France). These fiber-bundle probes provide faster image acquisition rates, and image streams can be merged by a technology called mosaicing. For a single probe the imaging plane is fixed, and resolution is limited by the number of the fibers (30,000 single fibers = pixels) (Fig. 1).

Contrast agents and staining protocols. For endomicroscopy, use of a fluorescent contrast agent that shows excitation and emission spectra within the laser light range used is mandatory. Most studies in humans have been performed with intravenous fluorescein sodium and blue laser light. Fluorescein is a nontoxic agent that has been used in ophthalmology for decades for retinal angiography and has also been shown to be safe for endomicroscopy (48). It quickly distributes throughout the body, and endomicroscopy is possible within seconds after injection. Fluorescein yields good contrast of vessel architecture and cellular and subcellular details. Alternatively, Acriflavine has been evaluated after topical administration. It predominantly stains nuclei and, to a lesser extent, cytoplasm (25). Because of its nuclear enrichment, concerns about potential mutagenicity have been raised; however, there are no reports on such adverse events, and this chemical compound has been used in disinfection solutions for decades. Topical cresyl violet has recently been shown to negatively render nuclei by predominantly staining the cytoplasm, yielding satisfactory contrast, although its excitation and emission spectrum is located at the border of the spectra of the presently used blue laser confocal devices (12). In animal studies with lower safety requirements, a multitude of staining protocols has been evaluated in vivo, and even antibodies have been fluorescently labeled (Table 1, Fig. 2). For all data represented in figures, ethical approval has been obtained from our local Institutional Review Board, and the confocal endomicroscope is CE certified and FDA approved.
Experimental Applications in Animals

Initial studies using in vivo confocal microscopy have mainly focused on visualizing morphology in healthy animals and diseases in vivo. Particularly, the use of intravenous Acriflavine yielded high-resolution images in vivo that were comparable to conventional hematoxylin and eosin staining, however, without fixation artifacts (9). In DSS colitis in rats, the topical application of fluorescein and acridine orange enabled in vivo imaging of early colitis even before macroscopic evidence of epithelial damage was visible (32). In mouse models of human liver diseases, confocal endomicroscopy with the handheld probe was able to discriminate mononuclear infiltration in models resembling human autoimmune diseases from granulocytic infiltration in a bile duct ligation model by resolution of the nuclear morphology of the infiltrating cells (using Acriflavine) and to delineate fatty liver disease (using fluorescein) (13).

Additional functional information could be gathered by in vivo imaging. Increased vessel permeability, which is a common feature of inflammation, was clearly identified by leakage of FITC-labeled dextran from liver lesions (13). In a different setting, perfusion abnormalities after thermal ablation of liver tissue have been described in vivo (38). This functional visualization of perfusion is significantly limited in most if not all ex vivo approaches, whereas for in vivo confocal microscopy the integrity of the tissue of interest itself is maintained. A step toward automated vessel analysis was recently validated, showing that neovascularization in malignant lesions can be detected using computer algorithms on the basis of vessel diameter distribution, density, volume fraction, and fractal dimensions of vessel structure (30). First evidence that this approach can be transferred to humans was described for Barrett’s esophagus (2) although further validation is warranted.

Dual and even triple labeling has been possible using a single channel instrument with the same excitation and detection wavelengths for all different stains. To establish the temporal relationship of morphological changes with microcirculatory dysfunction in a rat model of DSS-induced colitis, topical tetracycline and systemic FITC-dextran have been combined. Disruption of the gut epithelium and increased vessel leakiness were visualized simultaneously (33). For triple staining, visualization of blood plasma flow by FITC-dextran was combined with nuclear labeling using Acriflavine and targeted staining of erythrocytes. Although such an approach does not allow colocalization experiments at different wavelengths, the labeled target structures could be distinguished by their morphology and the relative fluorescence intensities of the contrast agents used in vivo by optimizing the concentrations of the individual fluorescent compounds (and restaining under direct microscopic monitoring) (11).
Upper Gastrointestinal Tract

Endomicroscopy of the esophagus. In patients, confocal endomicroscopy has been studied for noninvasive in vivo diagnosis of Barrett’s esophagus and squamous cell cancer of the esophagus. In Barrett’s esophagus, repeat random-quadrant biopsies have been advocated for screening for intraepithelial neoplasia (IN), but their diagnostic benefit is still not clearly defined. Endomicroscopy is able to visualize the specialized intestinal epithelium associated with Barrett’s esophagus and the key structure goblet cells in the distal esophagus (Fig. 3). Barrett’s epithelium shows a villous appearance with dark inclusions within the superficial lining, corresponding to mucin in goblet cells. Vessels in Barrett’s esophagus are clearly different from intrapapillary capillary loops in normal squamous epithelium.

Barrett’s-associated IN is characterized by a disorganized tissue structure. Malignant cells (with a more acidic cytoplasm)
usually appear darker because fluorescein shows pH-dependent signal intensity. By a simple-to-use classification on the basis of the tissue and vessel organization, the accuracy of endomicroscopy to predict the presence of Barrett’s esophagus and associated IN was over 90% (23) by using the integrated type of endomicroscopy.

In a study using probe-based endomicroscopy, a high-negative predictive value was demonstrated to rule out endoscopically invisible neoplasia, however, at the trade off of a low sensitivity (41).

In a follow up trial, scope-integrated endomicroscopy with only targeted biopsies almost doubled the diagnostic yield for overt and unapparent neoplasia compared with a standard random-biopsy protocol (6). It also significantly reduced the number of biopsies per patient, and two thirds of patients in the surveillance group did not need any biopsies at all. In squamous cell cancer, microscopic alterations could be visualized including the cellular disorganization and gradual vascular changes associated with cancerogenesis in vivo (4, 39).

Fig. 5. Confocal classification for endomicroscopy of the colon. A: healthy human colonic mucosa shows a honeycomb arrangement of normal crypts with goblet cells (arrows), which in deeper sections (B) are surrounded by capillaries (arrows). Erythrocytes can be seen as black dots within the fluorescein-filled, whitish vessel lumen. C: in inflammation (ulcerative colitis), crypts may show a slit-like or star-shaped openings in superficial sections. Acute inflammation often demonstrates a dense cellular infiltration with fluorescein leakage. D: in deeper mucosal layers in chronic inflammation, the increased vascularity is seen (arrows). Crypts are of irregular size and with increased distance from each other. E: in a colitis-associated lesion, endomicroscopy shows epithelium of villous appearance with different epithelial height. Note that the basal membrane is still intact. F: neoplastic vessels are of irregular diameter (arrows), and red blood cells often appear to be stacked within those vessels. Histopathology confirmed high-grade intraepithelial neoplasia. Scale bar = 100 μm.
**Endomicroscopy of the stomach and small intestine.** In the stomach, endomicroscopy was able to differentiate hyperplastic from adenomatous polyps with high accuracy (29). Endomicroscopy can also visualize Helicobacter pylori (21), gastritis (51), intestinal metaplasia (17), and gastric cancer (26). This is especially important because these conditions frequently occur side by side within the same patient, exemplifying the need to meticulously target biopsies to the region of interest by endomicroscopy (Fig. 4).

In celiac disease, sampling error is common and may generate false-negative results if biopsies are targeted by videoendoscopic appearance alone. Endomicroscopy was able to define villous atrophy and crypt hyperplasia in a large case series with excellent accuracy in vivo (27). These mucosal alterations were sensitive to change if patients were monitored with endomicroscopy on a gluten-free diet.

**Lower Gastrointestinal Tract**

**Endomicroscopy in screening colonoscopy.** The first clinical study on endomicroscopy was done in patients undergoing screening or surveillance colonoscopy (20). This initial study established a simple-to-use confocal pattern classification to differentiate normal from regenerative (i.e., inflammatory or hyperplastic) from neoplastic mucosal changes on the basis of crypt and vessel architecture with high accuracy (Fig. 5). These results were confirmed in follow-up trials (42). These studies provided the first published evidence that histopathology could be reliably predicted by gastroenterologists during ongoing endoscopy. Because fluorescein does not stain nuclei, some typical features of conventional pathology cannot be evaluated. However, when combined with topical Acriflavine, even the differentiation of low-grade from high-grade adenomas was possible (43). Indirect nuclear visualization was also obtained using cresyl violet for both chromoendoscopy for identification of colorectal lesions and endomicroscopy for their immediate differentiation (12).

**Endomicroscopy in inflammatory colitis.** In inflammatory bowel diseases, endomicroscopy serves two distinct needs, to establish the diagnosis and to diagnose complications. In ulcerative colitis (UC), patients are at increased risk to develop colorectal neoplasia. Because a large area has to be screened, endomicroscopy was combined with panchromoendoscopy of the colon in a prospective trial and compared with standard white-light endoscopy surveillance (22). Endomicroscopy predicted the presence of IN with an accuracy of 97.8%. This study provided further support of the concept that taking “smart”, targeted biopsies rather than random biopsies is advantageous; normal in vivo endomicroscopic architecture predicted normal histopathology in >99% of sites. Targeted biopsies from circumscribed areas with suspicious confocal pattern still yielded significantly more IN than a 10-fold higher number of random biopsies in patients undergoing white-light endoscopy. Inflammatory mucosal changes could be diagnosed in vivo as well (Fig. 5).

Microscopic colitis is a chronic intestinal inflammation that is frequently underdiagnosed by random specimens because of its patchy distribution throughout the entire colon. Endomicroscopy enables rapid acquisition of multiple optical (virtual) biopsies. Therefore, physical tissue sampling can be restricted to colonic sites of thickened subepithelial bands and cellular infiltration of the lamina propria (24, 36, 50). In a similar approach, patients with acute diarrhea after bone marrow transplantation were examined by endomicroscopy. In these patients, rapid diagnosis of graft-vs.-host disease (GvHD) is essential, but biopsies are associated with increased bleeding complications. Endomicroscopy with fluorescein and Acriflavine provided immediate diagnosis of GvHD and even enable visualization of epithelial apoptosis in vivo (3). Similarly, the endomicroscopic diagnosis of intestinal spirochetosis and amyloidosis have been reported (16).

**Current Developments and Future Challenges**

**Expanding fields of indication.** Applications for endomicroscopy are expanding rapidly and obviously fulfill a yet unmet scientific and clinical need for in vivo imaging. There is a strong trend to apply endomicroscopy to other organs apart from the gastrointestinal mucosa. Using a flexible probe fitted through the working channel of a duodenoscope, confocal imaging of intrabiliary neoplasia has been obtained (34). Although other than with the gastrointestinal mucosa, resolution does not (yet) quite match conventional histopathology using fluorescein as a contrast agent, and this study provided first evidence that microscopic imaging can augment diagnosis in a field with an undoubted high clinical

![Fig. 6. Near-infrared (NIR) light confocal microscopy of the liver. A: hepatocytes are visualized using indocyanine green as a contrast agent and NIR light penetrating through the capsule into the healthy human liver parenchyma. The parenchymal liver cells appear as perpendicular columns toward the central vein of a liver lobule. Some nuclei can be displayed as central clearances within the cytoplasm. B: in liver cirrhosis, the liver architecture is disturbed by fibrous bands (which do not take up indocyanine green and appear black, arrows). Some steatotic cells can be seen (arrowheads) with fat inclusions. Scale bars = 50 μm.](http://ajpgi.physiology.org/)

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demand. A sterilizable rigid confocal probe design has recently enabled microscopic imaging of the human liver during ongoing minilaparoscopy (10). Again, fluorescein-enhanced microscopic images provided lower image quality than conventional histopathology. However, sterile confocal intra-abdominal imaging was achieved for the first time. This type of intra-abdominal imaging is presently tested for indications such as screening of tumor resection margins in surgery but also in a Natural Orifice Transluminal Endoscopic Surgery approach (47). There is no rationale to restrict confocal imaging to the field of gastroenterology. Confocal endomicroscopy with the above mentioned systems has been evaluated in pilot trials for imaging of cervical intraepithelial neoplasia during colonoscopy, using Acriflavine (45), for imaging of neoplasias of the oral cavity (18), and for imaging of neoplasias of the urinary bladder (44).

Fig. 7. Confocal imaging using Acriflavine and cresyl violet. A: topical staining with Acriflavine in patients allows visualization of the nuclei of the epithelial layer by positive labeling (arrows). B: cresyl violet negatively renders nuclei at the basal side of the epithelial lining of the crypts (arrows). Scale bar = 100 μm.

Fig. 8. Gaps in the mucosa and bacterial translocation in Crohn’s ileitis. A: in a patient with Crohn’s disease in clinical remission, few gaps are found in the regular epithelium of the terminal ileum (arrows). As a sign of underlying inflammatory activity, some cells are found after having been shed (arrowheads) into the lumen. Note that the lumen shows whitish contrast as a consequence of fluorescein leaking from inflamed areas. B: in some of these gaps, bacteria can be seen as white dots (arrow) but also in the lumen. No bacteria are found in the lamina propria in this mild inflammation. C: in more severe terminal ileitis, a dense inflammatory infiltrate is found within the villus at the lower right corner. Bright fluorescein leaks from the vessels into the epithelium (arrow) but is still contained by an intact luminal lining, and only few cells have been shed (arrowheads). D: massive cell shedding (arrows) has resulted in almost complete disruption of the epithelium with massive shedding of cells and leaking of fluorescein into the bright white lumen (asterisk). Scale bar = 50 μm.
Increasing imaging plane depth. Most of the trials published so far evaluate alterations that arise from the epithelial layer of the organ of interest. However, the full depth of infiltration of a lesion can usually not be visualized, given the limited imaging plane depth obtained with the presently used blue laser light systems. Longer wavelengths such as near-infrared (NIR) light or nonlinear imaging techniques such as two-photon or multiphoton microscopy are frequently used in bench-top confocal microscopy. They show an increased imaging plane depth of up to 1 mm and require contrast agents compatible with the excitation wavelengths that are not yet available for human endoscopy. NIR confocal microscopy has already been tested for animal research (46). Recently, a first trial evaluated the use of NIR light for liver microscopy during minilaparoscopy in humans and found an improved resolution of liver microarchitecture by enhanced permeation through the thick liver capsule and deeper imaging plane depth (8). In this study, indocyanine green was used as a contrast agent (Fig. 6).

Alternative staining protocols. Another issue addressed in recent studies is adaptation of staining protocols to allow imaging of distinct tissue aspects. This is mainly correlated to the fact that fluorescein usually does not allow visualization of nuclei, and therefore evaluation of an important histological feature of neoplasia is not routinely achieved. Therefore, a combination of systemic fluorescein with topical Acriflavine has been tested. Differentiation of high-grade from low-grade IN in adenomas during colonoscopy has been obtained with such combined staining (43). In a pilot study on colorectal lesions in humans, topical cresyl violet, which stains the cytoplasm, yielded indirect information on nuclear morphology (Fig. 7) (12). It seems reasonable to speculate that future advancements in endomicroscopy technique will allow in vivo multiwavelength imaging at different wavelengths such as established in bench-top confocal microscopy. This will further enhance the microarchitectural issue that can be investigated in vivo.

Functional imaging. In vivo endomicroscopy in humans is less prone to tissue artifacts than routine histopathology of a biopsy specimen. In a translational approach, it has been demonstrated that the intestinal mucosa is punctuated by numerous microscopic gaps that arise from shedding of single epithelial cells at the mucosal surface (Fig. 8). Features of such gaps were established and differentiated from mucin inclusions by imaging in Math1−/− mice, which lack intestinal goblet cells. Epithelial barrier function is maintained despite these gaps (49). Such gaps seem to be more frequent in mouse models of colitis (25) and in patients with inflammatory bowel diseases even at mucosal sites that do not show any macroscopic signs of inflammation. In addition, enhanced cell shedding in these patients can be observed in vivo using endomicroscopy. These sites coincide with sites of enhanced bacterial translocation into the epithelium or lamina propria that can also be visualized with confocal endomicroscopy (37). These studies demonstrate the power of in vivo microscopic imaging to visualize physiological events in vivo on a microscopic basis and have the potential to induce a paradigm shift in our understanding of pathophysiology of gastrointestinal diseases.

Molecular imaging. Confocal endomicroscopy not only allows accurate resolution of tissue morphology and function but also enables molecular imaging. Molecular imaging comprises wide-field techniques for detection of lesions and microscopic techniques for in vivo immunohistochemistry. It is based on the molecular fingerprint of gastrointestinal pathologies. For fluorescent staining, exogenous agents such as peptides, antibodies, or probes with tumor-specific activation are fluorescently labeled (14, 31). In a rodent model of melanoma, malignant cells were detected with in vivo confocal microscopy by injection of FITC-labeled antibodies (1). In a similar approach, a carboxy-fluorescein-labeled ligand to somatostatin receptor (sstr) was synthesized. After systemic application to rats, internalization into sstr-positive pancreatic islet cells was recorded after an incubation time of 45 min, as confirmed by immunohistochemistry. Molecular in vivo targeting of sstr-positive tumor cells was demonstrated in mice (7). In orthotopic mouse models of pancreatic cancer, fluorescent targeting with probes, which were protease-activated in the tumor, allowed specific detection of pancreatic lesions at a cellular level (46). The tumor-specific fluorescent signal was significantly stronger than in normal pancreas or in acute pancreatitis.

Initial studies have translated molecular imaging to endoscopy in humans. During colonoscopy, adenomas have been detected using endomicroscopy after topical application of a labeled peptide derived from a phage library with preferential binding to human neoplasia (19). Such binding characteristics allowed visualization of neoplastic crypts, whereas nonneoplastic crypts remained unstained. Labeling of antibodies to the epidermal growth factor receptor (EGFR) has allowed selective visualization of EGFR-positive colorectal cancers in animal models (Fig. 9) and human tissue with the use instrument settings approved for clinical use (15). Such trials exemplify efforts to stratify patients immediately during endoscopy and to provide an individualized therapy on the basis of in vivo expression patterns, taking into account recent discussions about the validity of some ex vivo immunostaining techniques (40). They could also provide a tool for surveillance of re-

Fig. 9. In vivo molecular imaging of colorectal cancer. In a nude mouse xenograft model, human colorectal cancer cells were implanted. When FITC-labeled anti-epidermal growth factor receptor (EGFR)-antibodies were injected, the tumor showed a strong EGFR-specific fluorescence, indicating that confocal endomicroscopy is able to visualize the molecular signature of human cancer cells in vivo. No additional contrast agent was injected.
spouse to targeted therapy in the treatment of gastrointestinal cancer. In addition, molecular events could be studied in vivo in their natural environment.

Conclusions

In summary, endomicroscopy has for the first time provided in vivo histology during endoscopy with high resolution and subsurface imaging planes. Initial trials have been confirmed by follow up studies in a multitude of gastrointestinal diseases from both upper and lower gastrointestinal tract. However, endomicroscopy has gone beyond visualizing mucosal morphology and is now also used to interrogate cellular events in their natural environment. Together with molecular imaging, this opens new horizons for immediate diagnosis and management during endoscopy and also for clinical and basic science in gastroenterology.

DISCLOSURES

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REFERENCES


