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Review

Confocal Endomicroscopy: *In Vivo* Diagnosis of Neoplastic Lesions of the Gastrointestinal Tract

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Abstract. *Confocal endomicroscopy is a novel technology which allows subsurface histological diagnosis at a cellular and subcellular level in vivo. It thereby provides instantaneous histopathology during ongoing upper and lower endoscopy. This allows immediate diagnosis of neoplastic and inflammatory lesions of the intestinal mucosa. Studies have demonstrated the power of confocal endomicroscopy in screening and surveillance colonoscopy, ulcerative colitis, Barrett's esophagus, and gastric cancer. In animal models of human diseases, the same technology has provided molecular imaging of cancer, functional imaging of altered perfusion in malignant and inflammatory disease and high resolution in vivo morphological diagnosis. Fields of ongoing research are the development of molecular markers for in vivo immunohistochemistry and the application of confocal microscopy to intraabdominal organs in humans. Confocal endomicroscopy is evolving as a novel technique for rapid intravital diagnosis of gastrointestinal neoplastic diseases at the microscopic level and bears the potential for molecular imaging in humans in the future.*

Carcinomas of the upper and lower gastrointestinal tract have a poor prognosis, and curative treatment is most commonly only achieved in local disease. Therefore, early and rapid diagnosis is of paramount importance for the successful management of premalignant and malignant lesions. Management usually relies on histopathological evaluation of a tissue specimen as a gold standard of diagnosis. However, biopsy taking might be prone to sampling error, and tissue preparation for *ex vivo* analysis implies a time delay for definite diagnosis which is then not available during the endoscopic procedure. This potentially results in resection of a lesion that does not necessarily mandate invasive treatment, such as hyperplastic polyps (overtreatment) or in taking a biopsy instead of resection of a neoplastic lesion (undertreatment), subjecting the patient to unnecessary risks.

The implementation of endoscopic screening programs that aim at preventing cancer by resection of precursor lesions have been a major success for reducing the cancer rate. These programs mandate accurate diagnosis even of minute lesions. Kudo *et al*. stated in their well-known publication on the Pit Pattern Classification that "...the ability to establish an immediate endoscopic diagnosis that is virtually consistent with the histologic diagnosis has been the ultimate objective of endoscopists since the very earliest phases of the development of endoscopy" (1), alluding to their classification of colonic lesions by the chromoendoscopic staining pattern. However, they still had to rely on predicting the histopathology of neoplastic lesions rather than being able to actually see histopathology at a cellular level *in vivo*. This has recently become possible by the introduction of a novel technique termed confocal laser endomicroscopy. It allows magnification of mucosal structures of the gastrointestinal mucosa to a subcellular level by using confocal fluorescence microscopy during ongoing endoscopy, providing virtual optical biopsies *in vivo*. This new imaging modality has been successfully applied to predict neoplastic changes in the esophagus, stomach and colon in real time, allowing instantaneous targeted therapy.

Principle of Confocal Microscopy

Confocal microscopy is an adaptation of white light microscopy in which the light beam has to pass a system of two consecutive pinholes, before an image is detected. By this procedure, only light originating from a distinct in-focus imaging plane is able to pass those confocal lenses (pinholes), whereas out-of-focus light is geometrically rejected by the pinhole in front of the detector (2). Confocal microscopy therefore enhances spatial resolution compared

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with conventional fluorescence microscopy, especially in the axial direction. As a result, it permits observation of fine subcellular detail in conjunction with fluorescent labelling techniques or vital dyes, even in cells located many microns below the tissue surface (3). After the application of fluorescent agents, light emission is stimulated by laser excitation, and light returning to the detector is translated into a two-dimensional microscopic image. The *in vivo* applicability of such microscopic devices to humans (4) has been impeded by the need for a miniaturization of the former bulky bench top devices. Recently, a laser scanner for confocal laser microscopy has been miniaturized to meet the form and dimensions compatible with integration into a conventional endoscope.

Technique of Confocal Endomicroscopy

In the confocal endomicroscope (Figure 1), a miniaturized laser scanner has been integrated into the distal tip of a conventional colonoscope (Pentax EC-3870CIFK, Pentax, Tokyo, Japan). A solid state laser is flexibly connected to the endoscope delivering an excitation wavelength of 488 nm (blue laser light) *via* a single optical fibre. A lens system in the scanner focuses returning light onto the distal end of this single fibre which acts as a confocal pinhole. Light being emitted from other imaging planes does not focus on this point and thus does not blur the image to a significant extent. The microscopic field is raster scanned by the laser light, and the resultant signal is digitally translated into a two-dimensional grey-scale image that represents an en-face optical section through the tissue with a field of view of 475x475 μm. Maximum laser power output at the tissue surface is below 1 mW. Confocal frames are collected at a scan rate of 1.6 frames/s (512x1024 pixels) or 0.8 frames/s (1024x1024 pixels), approximating a 1000-fold magnification on a 19-inch screen. Optical slice thickness is 7 μm with a lateral and axial resolution of 0.7 μm. The range of the z-axis is from the surface to 250 μm below the tissue surface, permitting microscopic imaging across the mucosal layer. The submucosal layer cannot routinely be reached with the currently used laser wavelengths. Actuation of imaging plane depth within the tissue is controlled using two remote control buttons on the endoscope hand piece. Confocal and videoendoscopic images can be displayed simultaneously on two separate screens.

Fluorescein sodium is most commonly injected as a fluorescent agent (5, 6). It has been used for fluorescent retinal angiography in ophthalmology for decades, and only carries a very low risk of (allergic) side-effects. It yields cellular and subcellular details, connective tissue and vessel architecture at high resolution but does not stain nuclei. Acriflavine hydrochloride, an alternative fluorescent dye, is applied topically. It stains nuclei and subnuclear details, such as nucleoli, and cytoplasm to a lesser extent. Since it accumulates in the nucleus, it potentially carries a mutagenic risk. Although no such side-effect has been reported so far, its use should be carefully considered. Topical cresyl violet might overcome these concerns, but has only recently been tested for confocal endomicroscopy (7).

Confocal endomicroscopy is a technique that should be applied in a targeted fashion for characterization of suspicious lesions. For the screening of large mucosal areas, for example in the colonic mucosa in ulcerative colitis, combination with a technique for identification of circumscript lesions, such as chromoendoscopy is recommended (8). Lesions are then targeted under videoendoscopic control with the confocal imaging window which is slightly prominent from the distal tip of the endoscope and can be seen in the videoendoscopic image at the 7 o'clock position. Taking an optical biopsy from a lesion should always include consecutive sections from different imaging plane depths to achieve virtual volumetric (three-dimensional) tissue sampling. Interpretation of confocal images requires thorough knowledge of the mucosal architecture of the gastrointestinal tract. Therefore, close collaboration with an expert gastrointestinal pathologist should be sought during the learning process of *in vivo* endomicroscopy. The initial evaluation of confocal images should be achieved during the ongoing endoscopic procedure. It follows recognition of normal *vs.* pathological microscopic patterns based on the evaluation of the microvascular and crypt or glandular architecture rather than appreciating every subcellular detail. Confocal classifications have been proposed for lower (5) and upper (9) gastrointestinal disorders .

Esophagus

In Barrett's esophagus, the normal stratified squamous epithelium is replaced by villiform specialized columnar epithelium, and the annual rate of neoplastic transformation in Barrett's mucosa amounts to an estimated 0.5 % for an individual patient (10). Quadrant biopsies have been recommended for screening, but their diagnostic benefit is still subject to debate. Therefore, confocal endomicroscopy was prospectively evaluated for the diagnosis of specialized intestinal metaplasia and Barrett's associated intraepithelial neoplasia (IN) in 63 patients with long-lasting reflux symptoms, previously diagnosed Barrett's esophagus or scheduled endoscopic therapy for Barrett's associated neoplasias (9). Barrett's epithelium was diagnosed *in vivo* by the presence of villiform or glandular epithelium, the microscopic diagnosis of intrapapillary capillary loops and by the presence of goblet cells, which are pathognomonic for Barrett's epithelium and easily recognized by their dark staining mucin inclusion (Figure 2A). With conventional

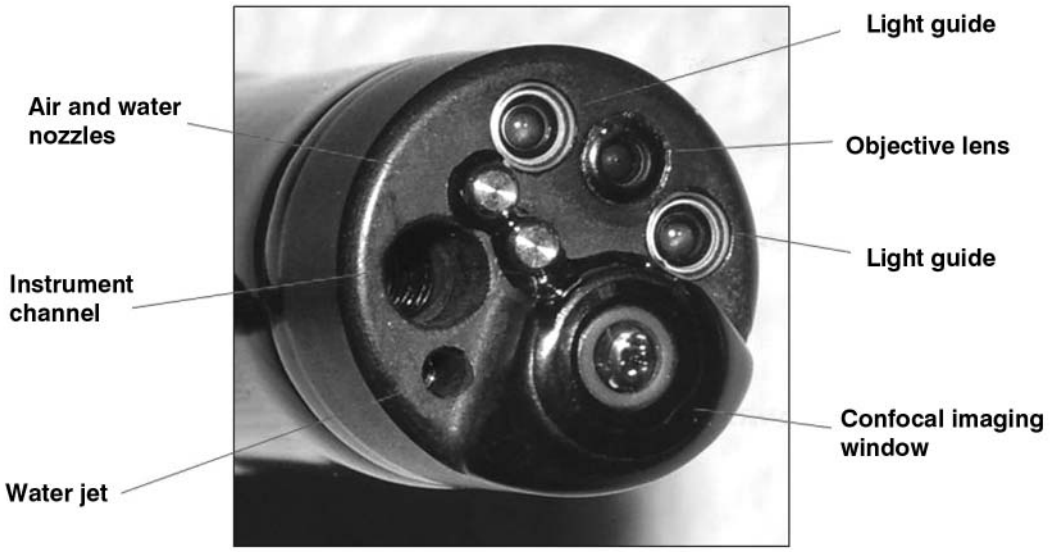

Figure 1. *In the confocal endomicroscope, a miniaturized scanning head has been intregrated into the distal tip of an otherwise conventional colonoscope. The confocal imaging window is slightly protruding from the tip and can be targeted onto a lesion under videoendoscopic control. Imaging depth is controlled using two additional buttons on the handpiece (FOV 475x475 μm).*

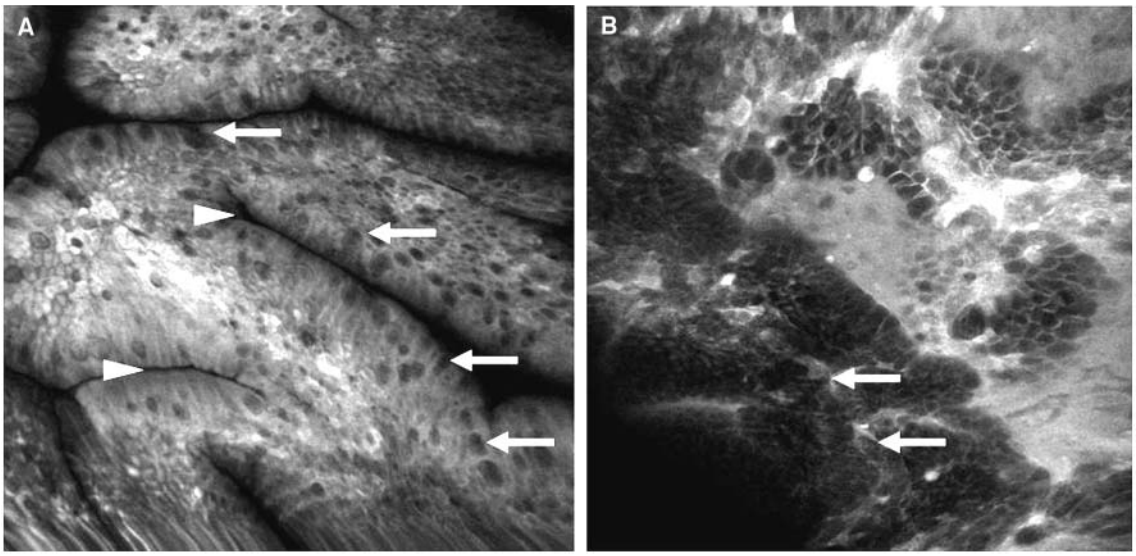

Figure 2. *Barrett's esophagus. Figure 2A: Barrett's epithelium is characterized by a villous like surface covered with high prismatic epithelium. Goblet cells are easily identified by dark mucin inclusions that are characteristic of specialised intestinal metaplasia (arrows). The opening of a Barrett's gland is clearly visible (arrowhead). Figure 2B: In Barrett's associated neoplasia, the tissue architecture is severly disturbed. Cells show a black appearance, and are no longer contained in the epithelial layer, but infiltrate through the basal membrane (arrow). Fluorescein from leaky tumor vessels permeates more easily into the lamina propria. Here, the infiltration into the lamina propria identifies alterations compatible with high grade neoplasia or early cancer even without nuclear staining (FOV 475x475 μm).*

histology from targeted specimens serving as the gold standard, prediction of specialized intestinal epithelium was possible with an accuracy of 96.8% by confocal endomicroscopy. In Barrett's associated IN, the monolayer of high prismatic cells was visibly disturbed, and in a subset of lesions, the basal membrane was interrupted by black cells that were not contained within the epithelial layer, indicating early stages of infiltration (Figure 2B). Accuracy of prediction of IN was 92.9% at 28 imaging sites *in vivo*. Tumor vessels were irregular and tortuous and showed an

enhanced leakage of fluorescein into the tissue. Inter- and intraobserver agreement was 0.84 and 0.89, respectively.

In a case report on squamous cell cancer of the esophagus, confocal endomicroscopy was a helpful tool for delineating the transition of normal intraepithelial capillary loops to pathological tumor vessels in the malignant lesion (11). The leakage of fluorescein from the tumor vessels into the surrounding tissue visualizes the increased permeability of pathological capillaries, adding a functional imaging quality *in vivo* to microscopic morphology.

Stomach

Early and accurate diagnosis is essential for successful management of gastric cancer and its precursor lesions, since the metastasis rate is as high as 18%, if submucosal involvement is present (12). In a recent trial by Yeoh and colleagues using confocal microscopy *ex vivo* after acriflavine application on biopsy specimens, typical features of gastric cancer were architectural atypia, increased nuclear to cytoplasmic ratio, and chromatin condensation (13). This allowed for a prediction of cancer with a sensitivity and specificity of 84% and 95%, respectively, and an interobserver agreement of 0.63. Even though fluorescein does not stain nuclei, in 15 patients with early gastric cancer and 3 patients with adenoma, the disorganized configuration of glands in the stomach was visualised after fluorescein injection as a prominent feature *in vivo*, rapidly identifying gastric neoplasia despite the fact that nuclei were not discernible (14). These encouraging results mandate further studies for *in vivo* detection and characterisation of early gastric neoplasia. In our own experience, a sudden break in gastric tissue microarchitecture should always raise the suspicion of gastric neoplasia (15). However, differentiation between adenoma and early intramucosal carcinoma is difficult due to the lack of ability to visualise nuclei after fluorescein injection.

In addition, confocal endomicroscopy has also been shown to visualise precursor lesions such as intestinal metaplasia. Probably secondary to an altered intracellular pH (fluorescein has a pH-dependent fluorescence intensity) metaplastic areas show a different staining pattern (brighter cells) and often contain goblet cells (dark inclusions within those brighter cells). By confocal endomicroscopy, larger areas of intestinal metaplasia can be microscopically screened *in vivo* by taking multiple optical biopsies to target the biopsy towards the most suspicious area.

H. pylori was classified as a human carcinogen in 1994 (16), potentially triggering the multistep progress of gastric carcinogenesis from intestinal metaplasia, chronic gastritis and intraepithelial neoplasia towards cancer (17, 18). *H. pylori* has recently been visualised *in vivo* by confocal endomicroscopy by its ability to incorporate acriflavine after topical administration (19). This not only exemplifies the power of confocal endomicroscopy to even visualise living bacteria during endoscopy *in vivo*, but also to show both the causative pathological agent (*H. pylori*, after acriflavine spraying) and its pathomorphological consequences (gastritis, after fluorescein injection) during ongoing gastroscopy. These *in vivo* findings can now be compared to the standard diagnostic procedures for *H. pylori* detection.

Screening and Surveillance Colonoscopy

The largest experience with confocal endomicroscopy has been gathered in colonoscopy (20). Here, both acriflavine and fluorescein have been evaluated for their ability to visualise the colonic and ileal mucosa (5, 6, 8). Since fluorescein allows easy, safe, and rapid microscopic visualisation across the whole range of the imaging depth and gives a good overall impression of the mucosal microarchitecture, it has been found more adequate for routine use in colonoscopy than acriflavine. In the first study to use confocal endomicroscopy in clinical practice, a total of 42 patients who were scheduled for screening or surveillance colonoscopy after prior polypectomy underwent video colonoscopy with confocal examination of suspicious circumscript lesions and standardized areas in the colon and terminal ileum (5). Confocal endomicroscopy was used in a targeted fashion on a total of 134 areas and flat, sessile, or polypoid circumscript lesions. A confocal pattern classification based on the cellular and crypt architecture combined with the microscopic appearance of intramucosal vessels was developed. Based on this easy-to-use classification, the distinction of normal mucosa from regenerative (hyperplastic or inflammatory) changes from neoplastic lesions was possible with high accuracy of 99.2%. Similar imaging results were obtained in a second study using the same confocal endomicroscopy system (6). These studies demonstrated for the first time that histopathology could be reliably predicted during ongoing colonoscopy (Figure 3). By confocal endomicroscopy, immediate endoscopic therapy of neoplastic lesions can be performed after *in vivo* microscopy. On the other hand, hyperplastic lesions which do not mandate endoscopic resection can be left untreated after endoscopic microscopic diagnosis, thereby optimizing diagnosis, therapy and associated risks during colonoscopy.

Ulcerative Colitis

Patients with longstanding ulcerative colitis are at elevated risk of developing colorectal neoplasia, and yearly colonoscopy is currently recommended in those patients. Chromoendoscopy has been shown to facilitate identification of flat lesions in high risk patients (21, 22). In a prospective randomized trial, 153 patients with ulcerative colitis in clinical remission scheduled for surveillance colonoscopy were

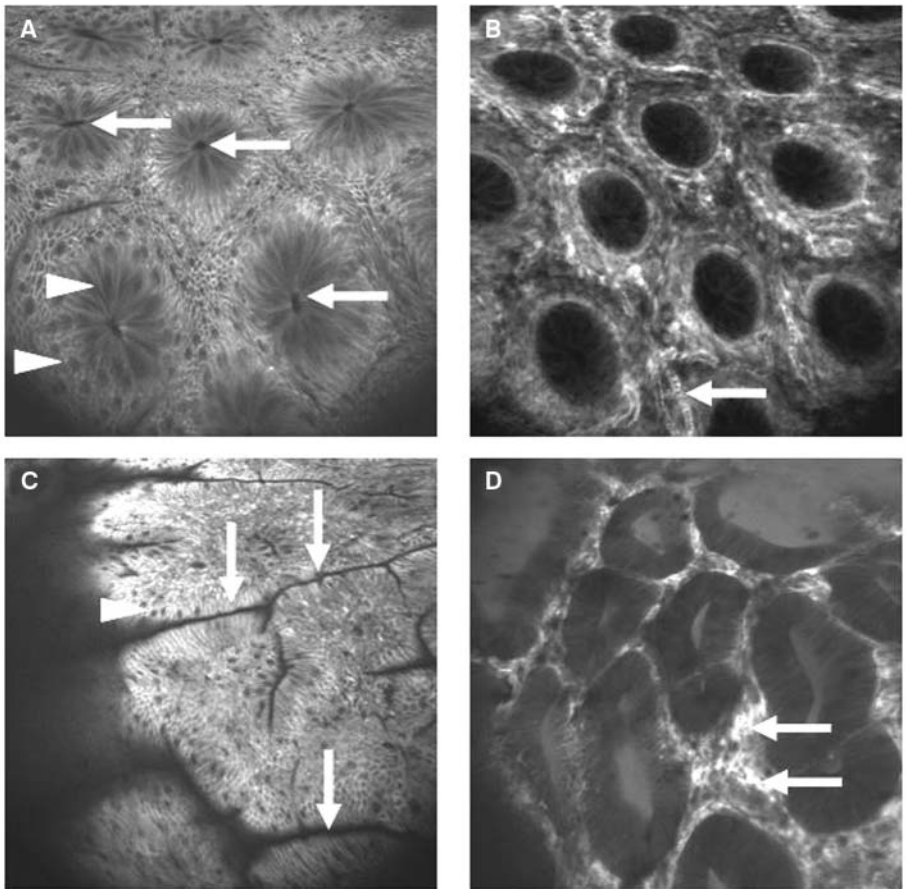

Figure 3. *Colonic mucosa: Figure 3A shows normal colonic mucosa with an en-face view onto the crypt openings (arrows). Those are regular, and the crypts hexagonal. Dark inclusions at the luminal side indicate mucin in goblet cells (arrowheads). In deeper sections (Figure 3B), the meshwork of capillaries is visualized. Each crypt is fed and drained by a circumventing vessel, perfused by fluorescent plasma after intravenous injection of fluorescein sodium. Black dots within the vessel lumina correspond to single red blood cells (arrows) and allow a rough estimation of the size of the vessel. In neoplastic tissue (adenoma, Figure 3C), the regular architecture is progressively lost. The surface appears villous, and crypts are elongated (arrows) or fuse laterally, corresponding to type III and IV pit pattern, respectively (1). Goblet cells (arrowheads) are rare. In deeper optical sections (Figure 3D), enhanced leakage of fluorescein is seen (arrows), indicating enhanced vessel permeability (FOV 475x475 μm).*

randomized at a 1:1 ratio to either conventional video white light colonoscopy or panchromoendoscopy with methylene blue (0.1%) in combination with confocal imaging. In this setting, chromoendoscopy was used to unmask lesions, and confocal endomicroscopy for immediate microscopic classification using the confocal pattern classification. *Ex vivo* histopathological correlation of targeted biopsies was performed according to the New Vienna Classification (23). While chromoendoscopy significantly enhanced the detection of flat lesions, as expected from prior studies, endomicroscopic prediction of the dignity of lesions was accurate in 97.8% (8). The time to perform a single complete colonoscopic evaluation was 31 minutes for video endoscopy, and increased to 42 minutes by adding confocal endomicroscopy and chromoendoscopy. Endomicroscopic guided colonoscopy resulted in an average of 21.2 biopsies per patient, while 42.2 biopsies were necessary in conventional colonoscopy. If only circumscript lesions had been biopsied, the number of biopsies could have been further reduced to 3.9 per patient without reducing the number of IN detected. Potentially as important, the negative predictive value for mucosa with a normal appearance on endomicroscopy not to contain IN was 99.1%. In this study, *in vivo* microscopically targeted biopsies increased the diagnostic yield of surveillance colonoscopy in ulcerative colitis patients. At the same time, this method allows the pathologist to focus on targeted biopsies from suspicious lesions only. This will probably significantly reduce the number of biopsies needed in the surveillance of patients with ulcerative colitis and will help to abandon the taking of untargeted random biopsies.

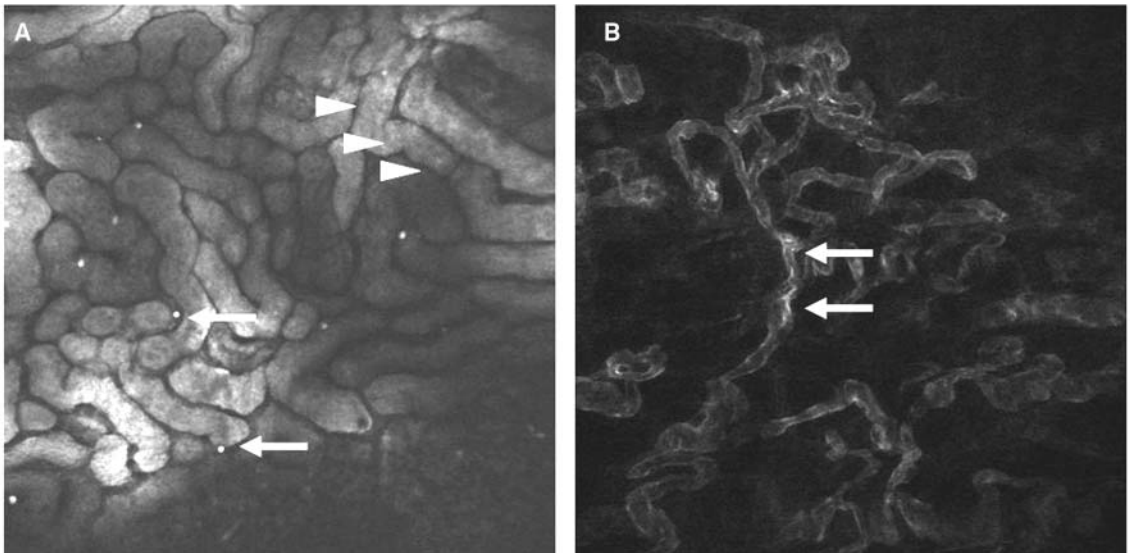

Figure 4. *By using a hand-held rigid probe (FIVE1, Optiscan Pty Ltd, Australia), confocal in vivo microscopy becomes possible in live animals. In Figure 4A, peripheral blood mononuclear cells (PBMC) were stained with acriflavine and reinjected. In vivo counterstaining with fluorescein shows bright PBMC (arrows) within the black capillary lumen around the renal tubules (arrowheads) in live mice. Figure 4B: In a tumor model (29), FITC-labelled L. esculentum lectin has been injected intravenously. This lectin binds to glycoprotein moieties on endothelial cells. Tumor vessels show a cork-screw appearance and irregular diameter. Due to an altered glycoprotein expression in tumor neoangiogenesis, the vessel wall contrast is patchy (arrow) and weaker as compared to non-tumor vessels (FOV 475x475 μm).*

In a similar approach, confocal endomicroscopy was used to differentiate sporadic adenomas in ulcerative colitis from dysplasia-associated lesions or masses (DALM). While sporadic adenomas can be treated by complete endoscopic resection, the presence of DALM with high grade dysplasia constitutes an indication for procto-colectomy. Confocal endomicroscopy was able to identify areas with dysplastic tissue around a lesion in DALM, whereas adenomas were surrounded by normal colonic mucosa. This finding potentially translates into immediate clinical consequences of resection *versus* biopsy and surgery (24).

Summary of the Current Position

Confocal endomicroscopy is a fascinating new technology that for the first time enables *in vivo* histopathology during ongoing endoscopy. This novel technique relies on fluorescent intravital staining that reveals morphology at a microscopic level. It should be applied in a targeted approach and can be combined with techniques for identification of suspicious lesions, which are subsequently immediately characterised. Confocal endomicroscopy requires training, just as any other new endoscopic technique, yet the first studies show encouragingly that endoscopists are able to evaluate virtual optical biopsies by means of simple and easy to apply classification systems, thereby targeting biopsies to the most relevant parts of a lesion and at the same time saving unnecessary random biopsies. Confocal endomicroscopy has been successfully evaluated in randomized trials for the detection of Barrett's esophagus and associated neoplasia, for the characterisation of neoplastic lesions in screening colonoscopy and in ulcerative colitis, and has shown its potential in other malignant entities such as gastric cancer and precursor lesions.

Future Perspectives

Current research aims at both broadening the applicability of *in vivo* confocal microscopy to different organs and at the same time enhancing the number of microarchitectural issues that can be addressed. By using a hand held rigid probe of a further miniaturised confocal microscopy system, *in vivo* microscopy has been evaluated in animal models of human hepatic diseases. Typical aspects of liver diseases (25) and liver metastases (26) were identified *in vivo* at a microscopic level. Based on these results, a sterilisable confocal microscopy probe has been introduced into the abdominal cavity during mini-laparoscopy in humans for *in vivo* microscopy of the liver (27). By this approach, the prediction of normal liver histology and of typical aspects of liver diseases has become feasible for the first time. This application also points to a potential use of intravital microscopy in surgery, for example for the intraoperative definition of tumor-free resection margins.

At present, only few compatible fluorescent agents (*e.g.* fluorescein sodium, acriflavine hydrochloride and cresyl violet) are registered for clinical use. Animal studies do not suffer this limitation, however, with thousands of fluorescent staining methods suitable for vital functional and molecular microscopy in living systems (Figure 4). For example, the use of metal nanoparticles and quantum dots coupled to antibodies has recently shown very promising results in the sensitive molecular characterisation of cancer cells by confocal bench top microscopy (28). Our group has recently added molecular and functional imaging to high resolution *in vivo* imaging using a further miniaturized rigid design of the confocal system used for flexible endoscopy (26). By synthesizing a fluorescently labeled high-affinity ligand to somatostatin receptors, endocrine cells of the rodent pancreas as well as malignant cells of neuroendocrine tumors could be specifically labelled and visualized by their molecular properties, providing the first evidence that molecular imaging by confocal microscopy can be achieved *in vivo* using a system compatible with application in humans.

Confocal *in vivo* imaging also offers the advantage of continuous imaging rather than taking "snap-shot" images. This can be used to monitor perfusion and vessel permeability changes by visualising leakage of fluorescent dye from capillaries, adding a functional quality and a time axis to morphology (29). Accurate observation of morpho-dynamic cellular events becomes possible in their natural environment, impacting significantly on basic and clinical science.

The identification of target molecules and the design of appropriate markers for use in animals and humans will now represent a major challenge of future research. In a murine model, a fluorescent peptide for the detection of colon cancer has been designed that was derived from a phage library (30). Other potential molecular targets are growth factors and their receptors or tyrosine kinases that are frequently over-expressed in malignant tissue. Their major role for tumor growth has been exemplified by the therapeutic efficacy of imatinib mesylate and epidermal growth factor receptor- or vascular endothelial growth factor-targeted antibody therapy. These findings may in the future be exploited to rapidly screen for potential response of cancer to biological treatment *in vivo*.

Instrument sensitivity and fluorophore quantum efficiency will be highly important parameters in the detection of minimal amounts of target structures that are ideally only present on target cells (*e.g.* malignant or inflammatory) to offer a highly specific cell identification (red flag technique). None of the currently available intravital staining protocols for clinical use fulfils the above mentioned requirements yet, and the development of specific molecular tracers still needs thorough evaluation as to specificity, sensitivity and safety in animal models, before application in humans is conceivable. However, having established the feasibility for *in vivo* microscopy, it is reasonable to speculate that rapid technological improvement will allow for similar technical features in confocal endomicroscopy as compared to bench top confocal microscopy. Currently used excitation wavelengths of endomicroscopy still limit imaging depth to the gastrointestinal mucosa. Intravital determination of submucosal infiltration would be highly desirable for immediate screening of endoscopic resectability. Here, exploitation of alternate laser lines and detection bands to achieve deeper tissue imaging (31) and co-localization of multiple fluorophores (multi-channel imaging) will undoubtedly enhance the power of endomicroscopy in the diagnosis of early neoplastic lesions in clinical medicine. In an experimental setting, *in vivo* confocal microscopy will extend the number of molecular biological and micro-architectural issues that can be investigated *in vivo*. Considering the time axis inherent to this *in vivo* procedure, this will result in a truly comprehensive four-dimensional morphological and molecular imaging. Confocal *in vivo* microscopy, therefore, not only facilitates diagnosis of cancer today, but also bears the potential to allow for a unique dynamic look into cellular life, disease and death *in vivo*.

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