TOPIC HIGHLIGHT

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Molecular confocal laser endomicroscopy: A novel technique for *in vivo* cellular characterization of gastrointestinal lesions

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focal microscope integrated into the distal part of an endoscope. By adding molecular probes like fluorescein conjugated antibodies or fluorescent peptides to this procedure (either topically or systemically administered during on-going endoscopy), a novel world of molecular evaluation opens up. The method of molecular CLE could potentially be used for estimating the expression of important receptors in carcinomas, subsequently resulting in immediate individualization of treatment regimens, but also for improving the diagnostic accuracy of endoscopic procedures by identifying otherwise invisible mucosal lesions. Furthermore, studies have shown that fluorescein labelled drugs can be used to estimate the affinity of the drug to a target organ, which probably can be correlated to the efficacy of the drug. However, several of the studies in this research field have been conducted in animal facilities or *in vitro*, while only a limited number of trials have actually been carried out *in vivo*. Therefore, safety issues still needs further evaluations. This review will present an overview of the implications and pitfalls, as well as future challenges of molecular CLE in gastrointestinal diseases.

Core tip: Confocal laser endomicroscopy (CLE) enables cellular visualization during on-going endoscopy. Lately, the method has been further refined by using fluorescent labelled molecular probes for estimation of receptors in carcinomas, illumination of subtle lesions and assessment of the affinity of drugs to specific lesions. This article presents the method of molecular CLE and gives a review of the current applications and drawbacks.

Abstract

While flexible endoscopy is essential for macroscopic evaluation, confocal laser endomicroscopy (CLE) has recently emerged as an endoscopic method enabling visualization at a cellular level. Two systems are currently available, one based on miniprobes that can be inserted *via* a conventional endoscope or *via* a needle guided by endoscopic ultrasound. The second system has a con-

INTRODUCTION

Flexible endoscopy is crucial in diagnosing and treating gastrointestinal disorders. The first fiber optical endoscopy was performed in 1955 and during the 1970's and 1980's the method became widely accepted and used for upper as well as lower gastrointestinal indications[1]. Flexible endoscopy is constantly undergoing technical refinements with chromoendoscopy increasing the ability of detecting subtle mucosal lesions. The main endoscopy companies have mimicked this technique by the introduction of narrow band imaging (Olympus, Tokyo, Japan), Fujinon intelligent color enhancement (Fujinon, Tokyo, Japan), and i-Scan (Pentax, Tokyo, Japan)[2]. However, a cellular evaluation is still needed to diagnose subtle mucosal lesions.

Confocal laser endomicroscopy (CLE), initially described in 2004, offers a magnification level of 1000-fold, which enables examination at a cellular level[3]. A CLE system consists of a laser source with a defined wavelength of 488 nm. The light is focused on a single spot at a precise depth in the mucosa and recaptured through a pinhole as all unfocused light is left out. As CLE is dependent on fluorescence, an intravenous (*iv*) fluorescent agent has to be administered prior to the investigation, with fluorescein being already approved as an off-label agent for gastrointestinal tract disorders. Alternatively, acriflavine can be administered topically during the procedure. CLE has been widely approved for identification of dysplastic lesions in Barrett's esophagus, for discriminating inflamed from malignant strictures in the common bile duct and for real-time pathological evaluation of colorectal polyps[4-6].

Currently, two CLE systems are clinically available. One is endoscope based (eCLE), where a confocal microscope is integrated in the tip of the endoscope (EC-3870CIFKTM, Pentax Tokyo, Japan). The resolution is high (1024 × 1024 pixels, with a lateral resolution of 0.7 μm) and the depth variable from 0 to 250 μm. The second system is probe-based (pCLE) and has various miniprobes available that all can pass through the working channel of the endoscope (esophago-gastric, colonic and cholangio miniprobes). Here, the resolution is somewhat lower and the depth is fixed at a certain level for the different miniprobes, but a high frame rate makes acquisition of movie sequences possible (8 frames/s). Very few head-to-head trials have been conducted comparing the two systems[7], but in daily practice the eCLE system has the advantage of a superior resolution and the variable

depth, whereas the pCLE system can be used on demand and has applications for the common bile duct and lately also for extraluminal applications, based on needle guided CLE (nCLE) fibers[8]. To perform CLE and interpret the images special training is required for the endoscopist as well as a close collaboration with a pathologist. When performed in a research setting, inter- and intra-observer variability can be estimated by correlating the CLE results with standard histology from biopsy specimens.

As described, CLE is able to provide a pathological diagnosis based on the morphology appearance of the tissue. Finally, molecular imaging of mucosal lesions has been added using different molecular probes in conjunction with CLE equipment (mCLE). Several techniques have been applied, all of them aiming at characterizing the examined lesions on a molecular level and thereby providing new information in real-time during the endoscopic procedure. Although numerous questions are to be answered in relation to these techniques, the method has the potential to evolve into a whole new area of research, ultimately leading to individualization of anti-angiogenic or anti-inflammatory treatment (tailored therapy). If a given agent is aimed at a specific cellular receptor in a given lesion, a detection and estimation of the representation of the receptor could possibly be correlated to the efficacy of the agent enhancing individual treatment.

This review will focus on the different techniques used for mCLE in the gastrointestinal tract, and will give an overview of the possible molecular and gastrointestinal targets (Table 1).

REVIEW CRITERIA

PubMed, Embase and Web of Science were searched using the terms "confocal laser endomicroscopy" or "endomicroscopy" or "mCLE" and "molecular". Only gastroenterological full-text papers using CLE were included. Abstracts until United European Gastroenterology Week 2012 and Digestive Disease Week until 2013 were searched, but only abstracts, which could contribute to this paper were included.

RESEARCH

The idea of molecular imaging during endoscopic examination is not new. During the late 90s several groups worked intensively at this aim introducing fluorescence, autofluorescence or laser induced endoscopic methods. Thus, in 2003, fluorescein-labelled monoclonal antibodies against carcinoembryonic antigens could be identified examining colonic neoplasias with conventional colonoscopy equipped with a narrow-band filter[9,10]. The trial was *in vivo* and no adverse events or immunological side effects in relation the topical administration of the monoclonal mouse antibodies were registered. After the introduction of CLE, the method of using targeted probes evolved, and the use of fluorescent-labelled antibodies in combination with CLE grew rapidly. The principle is that a

Table 1 Different techniques used for confocal laser endomicroscopy in the gastrointestinal tract

Ref.	Design	No	Target	Composite	Administration	CLE system
Hsiung *et al*[11]	*In vivo*	26	Colorectal adenomas	Fluorescein-labelled septapeptides	Topical	Probe-based
Foersch *et al*[18]	Animal, and *ex vivo*	25 and 14	CRC	Alexa-Fluor 488-labelled VEGF-antibodies	Intravenous and topical	Endoscope-based
Goetz *et al*[10]	Animal and *ex vivo*	68 and 16	CRC	FITC-labelled anti-EGFR-antibodies	Intravenous and topical	Endoscope-based
Foersch *et al*[37]	Animal and *ex vivo*	12 and 4	CRC	Alexa-Fluor 488-labelled bevacizumab	Intravenous and topical	Endoscope-based
Liu *et al*[23]	*In vivo*	37	CRC and adenomas	Alexa-Fluor 488-labelled anti-EGFR-antibodies	Topical	Endoscope-based
Cârţână *et al*[27]	*Ex vivo*	4	CRC	Alexa-Fluor 488-labelled anti-CD31-antibodies	Topical	Endoscope-based
Nakai *et al*[19]	Animal	2	Healthy esophageal and gastric mucosa	Fluorescein conjugated anti-EGFR-antibodies and anti-survivin-antibodies	Submucosal or topical	Probe-based
Hoetker *et al*[32]	Animal	26	Gastric cancer	FITC-labelled anti-EGFR1-antibodies or Alexa-Fluor 488-labelled cetuximab	Intravenous	Endoscope-based
Nakai *et al*[20]	Animal	2	Healthy pancreas	FITC-labelled anti-EGFR1-antibodies or Alexa-Fluor 488-labelled cetuximab	Intravital	Needle-based
Atreya *et al*[34]	*In vivo*	25	Colonic mucosa in Crohn's disease	FITC-labelled adalimumab	Topical	Endoscope-based
Neumann *et al*[26]	*Ex vivo*	2	Colonic mucosa in Clostridium difficile associated colitis	Fluorescein-labelled Clostridium difficile specific probe	Topical	Endoscope-based
Li *et al*[22]	Animal and *ex vivo*	20 and 23	Gastric cancer	Alexa-Fluor 488-labelled anti-MG7-Ag-antibodies	Intracardial and topical	Endoscope-based
Goetz *et al*[33]	Animal	44	CRC	FITC-labelled anti-EGFR-antibodies or Alexa-Fluor 488-labelled cetuximab	Intravenous	Endoscope-based
Neumann *et al*[25]	*Ex vivo*	N/A	Barrett's esophagus	FITC-labelled Muc2-antibodies	Topical	N/A
Sturm *et al*[12]	*In vivo*	25	Barrett's esophagus	FITC-labelled peptides	Topical	Probe-based

CRC: Colorectal carcinomas; FLUOS: 5(6)-carboxyfluorescein-*N*-hydroxy-succinimide ester; CEA: Carcinoembryonic antigen; VEGF: Vascular endothelial growth factor; FITC: Fluorescein isothiocyanate; EGFR: Epidermal growth factor receptor; N/A: Not available; CLE: Confocal laser endomicroscopy.
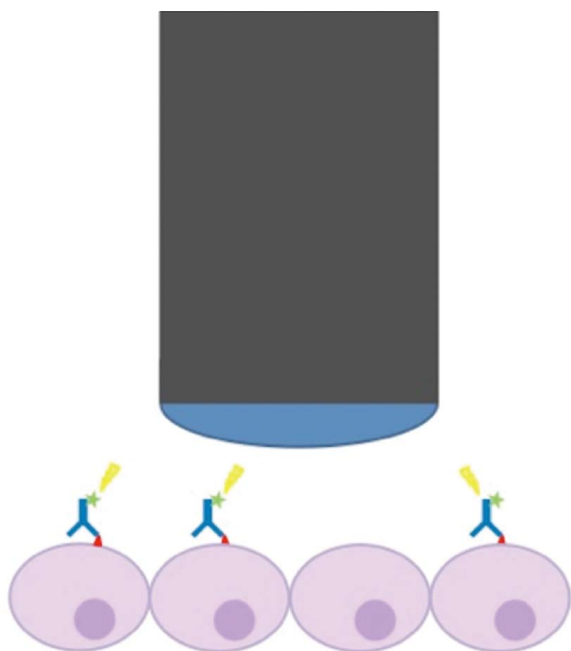
Figure 1 Molecular imaging with labelled antibodies using the confocal laser endomicroscopy system. Antibodies (blue) labelled with a fluorochrome (FITC or Alexa-Fluor 488) (green) bind to a specific target (red triangles) expressed on the plasma membrane of cells in the mucosal layer in the gastrointestinal tract (purple). The confocal laser endomicroscopy (CLE) scope (black and blue tube) excites the fluorochrome with a defined wavelength of 488 nm (yellow). Light is subsequently emitted and detected by the CLE scope.

fluorescent agent is conjugated to a probe aimed at a target molecule of interest. This composite can either by applied directly in the lesion, topically or systemic. Antibodies have been widely used; they have high specificity and are easy to label with a fluorescent agent (Figures 1 and 2). Main problems are the immunogenic nature of antibodies, long half-life in serum and slow penetration into diseased mucosa due to their high molecular weight. In addition, these will not cross the plasma membrane unless a permeabilizing agent is used. Thus, antibodies are mainly used for detection of membrane-associated proteins. Furthermore, antibodies are expensive to produce in high amounts. To overcome these problems, specific peptides can also be used. Peptides are easy to produce in large amounts, thus reducing production cost. Identification of a targeted peptide by phage display with preferred binding to premalignant colonic tissue and subsequent detection with CLE *in vivo* was initially reported by Hsiung *et al*[11]. The identified peptide showed high specificity towards the malignant cells. Since binding and selection of the peptide was verified by intact tissue panning, the specific target was unknown[11]. A recent paper published by Sturm *et al*[12] took this technique further and evaluated a peptide with specific binding affinity towards human esophageal neoplasia in patients with high specificity and no adverse effects. The authors have preliminary identified the target using mass spectrometry. Reports
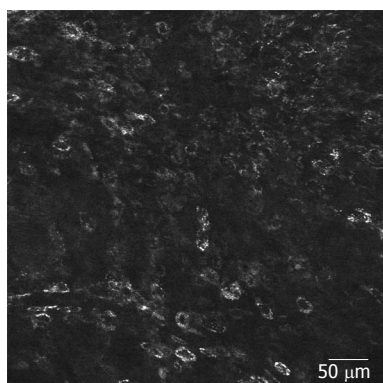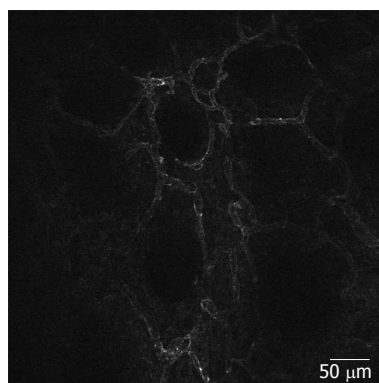
Figure 2 *In vivo* **epidermal growth factor receptor staining in a human colorectal cancer xenograft (SW480) after intratumoural injection of fluorescein isothiocyanate-labelled anti-epidermal growth factor receptor-antibody.** The edge length is 475 μm. Courtesy of Dr. M. Hoetker and Prof. M. Goetz, Tuebingen, Germany.

Figure 3 Molecular confocal laser endomicroscopy staining with Alexa-Fluor 488 labelled anti-CD31 antibodies. As CD31 is an endothelial marker, the structures seen in the image are microvessels in a colonic adenocarcinoma.

describing other probes such as aptamers, affibodies and nanoparticles are emerging and we are probably just witnessing the beginning of this era[13-16].

CHARACTERIZATION OF NEOPLASMS

One of the obvious applications of mCLE is the opportunity of a precise molecular characterization of a certain lesion, which can possibly lead to individualization of treatment. While Muldoon *et al*[17] presented a study estimating the expression of human epidermal growth factor receptor 2 (HER-2) and epidermal growth factor receptor (EGFR) receptors in cell cultures using a fiber-optic miniprobe based microendoscopy system constructed by the research group, Goetz and colleagues presented the technique using the eCLE system, which is commercially available today[10]. Using intravenously administered fluorescein isothiocyanate (FITC)-labelled anti-EGFR antibodies in a human-mouse colorectal carcinomas (CRC) xenograft model, a differentiation of the EGFR expression in tumours could be estimated. Furthermore, it was demonstrated that the technique could be used for distinction between neoplastic and non-neoplastic human tissue, when applying the labelled anti-EGFR antibodies topically to human colonic specimens *ex vivo*. For imaging, a rigid handheld CLE endoscope (FIVE1, Optiscan, Australia) was used. The protype has the same imaging characteristics as the eCLE systems, but is easier to use in animal and *ex vivo* experiments[10]. Another possible therapeutic target is vascular endothelial growth factor (VEGF) where a trial applying a similar technique as previously described demonstrated that molecular imaging was possible in murine tumour, xenograft models as well as in human surgical CRC specimens. The results were controlled by immunohistochemistry (IHC) and fluorescence microscopy[18].

A small study including two healthy pigs assessed the distribution of EGFR receptors and survivin in the esophagus and the gastric mucosa using pCLE[19]. Another study from the same group, evaluated whether a simi-lar method was feasible using needle-based confocal laser endomicroscopy (nCLE) for extraluminal investigation of the pancreas in conjunction with topical administration of anti-human EGFR-fluorescein conjugated monoclonal antibodies and anti-human surviving-fluorescein conjugated monoclonal antibodies. Although the number of pigs was limited, the technique was feasible[20]. However, resolution of the pictures obtained is rather low and there are no specificity controls shown for the EGFR staining. In general, the experience of mCLE using pCLE and nCLE is limited, mainly due to the relatively low resolution of the system, especially when applying the nCLE fibers.

MG7-antigen is a recently identified tumour-associated antigen, which is expressed in the majority of gastric carcinomas being related to a worse outcome when it is positive[21]. An estimation of the MG7-antigen could be made using xeno-models and surgical specimens, when fluorescently labelled MG7 antibodies were administered in the lesion and subsequently examined with CLE[22]. In this way, a possible predictor of worse outcome can be estimated during the initial investigation of the gastric lesion. Moreover, in case of a lesion with a large tumour surface, biopsies can subsequently be targeted to avoid false negative histology.

All of the procedures mentioned in the above were conducted either in animal models or in human tissue *ex vivo*, but in 2012 Liu *et al*[23] performed the technique *in vivo*, demonstrating that topically applied Alexa-Fluor 488 conjugated anti-EGFR monoclonal mouse antibody, could facilitate an estimation of EGFR expression in CRC or adenomas using CLE. And most important, no immunological adverse events were registered in the 37 patients included and no human anti-mouse antibodies were found in the serum samples taken from a subgroup of patients 4-6 wk after the procedure.

IMPROVEMENT OF DIAGNOSTIC ACCURACY

Morphology can be assessed by CLE, but discrimination

between microscopic changes could be further improved by adding a molecular staining. In Barrett´s esophagus the mucosal changes can be spread over a long segment and practically be invisible. Sturm and colleagues sprayed a FITC-labelled protein specific for esophageal neoplasia on the Barrett's mucosa while performing a segment gastroscopy with CLE[24]. The procedure was *in vivo* and when using this technique the sensitivity and specificity for neoplastic lesions in Barrett's and squamous epithelium was found to be 75% and 97%, respectively[12]. There were no adverse events in relation to administration of the protein. Similar results were reported using the FITC-labelled MUC2-antibody, which is only expressed on the surface of goblet cells. The examination was made on biopsy specimens and in this trial, the accuracy for detection of Barrett's esophagus was 97.2%[25]. If these results can be reproduced *in vivo*, the diagnostic accuracy of the examination could improve significantly.

In an *ex vivo* study on colonic biopsies adding a specific *Clostridium difficile* (*C. difficile*) probe, it was found that the bacteria could be localized and identified. Whether this trial can improve the diagnostic accuracy, when investigating inflamed colonic mucosa with the suspicion of *C. difficile* infection remain to be seen[26]. In our group, we used fluorescently labelled anti-CD31 antibodies, an endothelial marker, and CLE for identification of vessels in colorectal adenocarcinomas (Figure 3)[27]. The development of new vessels plays a vital role in carcinogenesis and we could clearly visualize the vessels within the tumours and estimate their shape and vascular density within the tumours in comparison with normal tissue. The trial was performed *ex vivo* and the technique can be used not only for discriminating neoplasia from healthy tissue, but possibly also for monitoring antiangiogenic treatment.

ASSESSMENT OF THE AFFINITY TO SPECIFIC DRUGS

Cetuximab is a monoclonal antibody that specifically blocks the extracellular component of the EGFR receptor and is currently being used in the treatment of colorectal neoplasms and in selective cases of gastric carcinomas[28-31]. In two trials investigating the technique using cetuximab treatment and cetuximab conjugated to Alexa-Fluor 488 in murine xenograft models with human CRC or gastric cancer, it was found that an estimation of expression was possible using CLE. Moreover, in the CRC trial, it was found that the tumours with a strong signal for cetuximab had slower tumour progression and longer survival. In other words, it was shown that in CRC as well as in the gastric cancer trial, an early response to treatment could be predicted[32,33].

The technique of conjugating drugs and estimating the affinity to a specific target can possibly have a major impact on treatment stratification. In a clinical phase 1 trial, Atreya *et al*[34] used topically applied FITC-labelled adalimumab in Crohn patients naïve to adalimumab therapy and found a strong correlation between the affinity of

the drug to the membrane bound TNF-alpha receptors in the bowel and the efficacy of the therapy. The procedures were *in vivo* and no serious adverse events were reported in the 25 patients included in the study. Although only published in abstract form, the preliminary results from this trial are promising, as the study is not only aimed at evaluating the feasibility of the method, but also at the ability of predicting the outcome of a given therapy.

Bevacizumab, an antiangiogenic drug aimed at the VEGF receptor, is commonly used in CRC treatment[35,36]. After conjugating the drug to Alexa-Fluor 488 and injecting it in a CRC xenograft model or topically applying it on fresh biopsy specimens from human CRC patients, the VEGF expression could be assessed using IHC as a gold standard[37]. These results are only preliminary, and the final results from the study will show if a strong CLE signal can predict the outcome of the bevacizumab therapy in CRC models or human.

PITFALLS AND FUTURE CHALLENGES

In order to implement mCLE, some considerations are needed. Tumour cells may have higher membrane permeability since cancer cells often have impaired plasma membrane integrity. Thus, the targeted antibodies may possibly penetrate the plasma membrane in the tumour cells if there is an intracellular localization of the protein of interest. In addition to expression from the plasma membrane, mCLE will also detect binding of the labelled antibody inside the tumour cells and thus generate a higher signal, which is not due to higher membrane associated expression of the protein. Another consequence of a raised permeability is the possibility of triggering systemic immunological side-effects when topically administering antibody conjugates. Only a limited number of trials have been carried out *in vivo*, therefore attention should be drawn continuously to possible immunological adverse events.

Furthermore, it is crucial that trials using targeted antibodies have proper controls such as blocking peptides and isotype controls, in order for mCLE to have a clinical impact. Although it seems to be a minor problem, registration for autofluorescence before adding fluorescein-labelled probes must also be required, when examining tissue. This can be easily taken into account by establishing a proper cut-off for the intensity of the laser light, which has to minimize tissue autofluorescence. The interpretation of the images obtained by mCLE can be challenging, thus the endoscopist would benefit from experience with conventional CLE as well as a close collaboration with a pathologist.

The administration of peptides seems safe in the studies published so far[11,12]. While the designed peptides have a high specificity and affinity for the lesions, it is a drawback that the precise binding site was unknown in the work published by Hsiung *et al*[11]. The report recently published by Sturm *et al*[12] preliminarily identified the target protein for the administered peptide and the results are very promising. Thus, designed peptides may very

well be the futures probe. Future studies will have to address this issue.

CONCLUSION

Molecular confocal laser endomicroscopy is a novel method that allows for visualization of cellular processes in real-time by combining a variety of either molecular probes or peptides with fluorescent *ex vivo* or in animal models. However, the method has recently also shown feasible for *in vivo* human applications and a huge potential for both diagnostic as well as therapeutic gastrointestinal applications has opened up in the near future. Estimation of VEGF and EGFR in colorectal and gastric carcinomas may be used for tailored oncological treatments as well as for prediction of treatment outcomes of biological therapy in inflammatory bowel disease. Further studies are needed to ensure the safety and to establish the final indications of mCLE.

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