

Systematic Intraoperative Application of Confocal Endomicroscopy for Early Detection and Resection of Squamous Cell Carcinoma of the Head and Neck

A Preliminary Report

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Objective: To use intraoperative confocal endomicroscopy for early detection and resection of squamous cell carcinoma (SCC) of the head and neck. A preliminary report.

Design: Prospective case series.

Setting: Tertiary referral hospital.

Patients: Fifteen consecutive patients with SCC of the oral cavity, hypopharynx, and larynx were included from the Department of Otolaryngology–Head and Neck Surgery, HSK Dr Horst Schmidt Kliniken GmbH, Wiesbaden, Germany

Interventions: Confocal endomicroscopy was performed during diagnostic and therapeutic procedures with a prototype of a rigid laser endoscope in combination with the already available technology of autofluorescence.

Main Outcome Measures: Real-time visualization of cellular and subcellular details during endoscopy. Diagnostic scores were applied to differentiate dysplastic and malignant mucosal changes of SCC of the head and neck from normal squamous cell mucosa using this method. Results were correlated with the well-established gold standard, histologic analysis.

Results: Dysplastic and malignant changes of head and neck squamous cell mucosa were endoscopically determined by this unique in vivo application of confocal laser endomicroscopy using a rigid probe.

Conclusions: We present preliminary and descriptive data using this novel technology in vivo. Considering the impact of a virtual real-time histologic analysis, this technology points to a very promising development. It may carry potential for quicker intraoperative diagnosis, less need for multiple frozen sections, and more precise resection margins.

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HISTOLOGICALLY, SQUAMOUS cell carcinoma of the head and neck (SCCHN) represents more than 90% of all malignant neoplasms of the head and neck. It can arise in various anatomic regions (eg, oral cavity, oropharynx, larynx, hypopharynx) and is associated with known risk factors like tobacco smoking and alcohol abuse, but if diagnosed early, it is curable.¹ The diagnostic gold standard for SCCHN is endoscopic evaluation combined with biopsy or excision followed by histopathologic examination. The treatment in the early stages is complete excision. Since differentiation of precancerous and invasive lesions can only rarely be made by inspection, especially in the margins of a lesion, the surgeon needs to take multiple selective biopsy specimens to determine the resection margins of a lesion. This often leads to a considerable de-

lay in obtaining definitive information and results in biopsies of healthy mucosa. Depending on the site of the lesion in the head and neck, extended resection can lead to significant functional deficits such as long-lasting aspiration and voice disorders. Thus, *overtreatment* (resection and biopsy of healthy mucosa) or *undertreatment* (biopsy instead of resection for neoplastic tissue) can lead to unnecessary risks and may compromise functional and oncologic outcomes for the patient.

Therefore, a number of interdisciplinary study groups are focused on achieving real-time information on dysplastic or invasive mucosal alterations and lesions during endoscopic procedures. Information on cellular and subcellular details, especially of margins of a lesion at the time of the endoscopic examination, could lead to an immediate definitive surgical treatment and additionally yield more information on the surrounding tissue (ie, mul-



Figure 1. Prototype of the rigid endomicroscope used for confocal endomicroscopy in a case of laryngeal carcinoma. A and B, An overview of the supporting technology. C, The probe itself has a working length of 320 mm and an outer diameter of 6.3 mm.

tifocal lesions that might be present in different mucosal areas, aka *field cancerization*).²

Laser-scanning confocal endomicroscopy is a diagnostic tool that is well suited for the visualization of microscopic structures in tissues. The technique allows for the assessment of changes in vascular architecture, connective tissue, and cellular components of the mucosa *in vivo*. The evolution of this technology over the past several years has led to the integration of a miniaturized laser scanner into the tip of a conventional flexible video endoscope that can visualize the mucosal details at subcellular resolution during endoscopy. This technique was established in the gastrointestinal (GI) tract by researchers of our group³⁻⁹ using a flexible confocal endomicroscope in detecting different inflammatory and neoplastic abnormalities in the human colon, esophagus, and stomach.

In a pilot study, our group¹⁰ investigated the feasibility of the method using the flexible endoscope in different regions of the human oral cavity and the oropharynx after intravenous application of fluorescein sodium. We were able to show for the first time to our knowledge that confocal endomicroscopy is suitable for evaluation of epithelial linings of the buccal mucosa, tongue, and floor of the mouth. Signs of malignancy (disturbed tissue architecture and increased density of blood vessels with irregu-

lar, elongated, and enlarged appearance) could be identified *in vivo*. In addition, adding topical acriflavine hydrochloride as a contrast agent on *ex vivo* specimens allows identification of prominent and irregular nuclei with increased rates of mitosis. This proof-of-principle investigation made clear that targeting of the lesion with the tip of the endoscope and the stable and precise positioning of the flexible instrument can be challenging around the narrow anatomic sites of the head and neck.

Owing to limitations imposed by the form of the flexible equipment and by sterilization workflows, the use of the flexible technique has been limited to the gastrointestinal tract so far. However, a prototype of a rigid miniaturized probe for confocal endomicroscopy of the human liver has been developed by members of our group and already evaluated concerning the feasibility in a clinical investigation in humans.^{11,12} Confocal imaging with this rigid probe allowed *in vivo* microscopic analysis of healthy and diseased human liver for the first time to our knowledge during ongoing minilaparoscopy in humans.

In the present study, we applied for the first time to our knowledge the new prototype of a rigid confocal laser endomicroscope to the systematic intraoperative evaluation in the head and neck. In this study, we also applied this new prototype in combination with the already available technology of autofluorescence, which is suitable for highlighting areas of interest in a more specific way (“red flag”). Highlighted mucosal areas were then analyzed on a microscopic level by endomicroscopy in a complementary fashion. We present herein our initial experience and the present state of development in this technology of rigid confocal laser endomicroscopy in the field of head and neck surgery.

METHODS

We used a novel rigid laser endoscope to evaluate 15 patients with SCCHN at a tertiary referral center (Department of Otolaryngology–Head and Neck Surgery, HSK Dr Horst Schmidt Kliniken GmbH, Wiesbaden, Germany). Written informed consent was obtained from each patient before examination. Excluded were women of childbearing age, patients younger than 18 years, patients with an allergy or adverse reaction to fluorescein, and those unable to give informed consent.

The new prototype device is a rigid, handheld probe (Optiscan) (**Figure 1**) that achieves the same optical properties as the flexible confocal endomicroscope, which was validated for the GI tract in the past.^{11,12} It uses a single optical fiber acting as both the illumination point source and the detection pinhole, allowing the miniaturization required for endoscopy. During examination, the plane depth visualized from the surface to a maximum of 250 μm can be controlled using 2 remote control buttons on a foot switch. During laser endoscopy, a solid-state laser delivers an excitation wavelength of 488 nm at a maximum laser power output of 1 mW or lower at the surface of the tissue. Images were obtained from adjacent imaging planes in different tissue depths, successively increased by 4- μm increments. Confocal image data were then collected at a scan rate of 0.8 frames/s (1024 \times 1024 pixels), using an optical slice thickness of 7 μm . Lateral resolution was 0.7 μm . The field of view was 475 \times 475 μm . Images were displayed in real time on a 19-inch screen at a magnification approximating \times 500. The

maximum tissue penetration depth of endomicroscopic laser scanning was 250 μm . This allowed imaging of the mucosal layer, including epithelial cells and the lamina propria.

Patients with SCC of the oral cavity ($n=4$), oropharynx ($n=7$), hypopharynx ($n=2$), or larynx ($n=2$) were included. Endomicroscopy was performed during diagnostic and therapeutic procedures following a standard protocol. During resection of lesions, images were generated from the tumor site as well as from macroscopically normal mucosa at defined distances from the margins of resection.

To enhance the naked-eye judgment on localization and extent of a suspect lesion, autofluorescence was applied as an initial step (TRICAM PDD Three-Chip Camera with SL II Control Unit, D-Light C/AF system light source, Hopkins straight-forward telescope-endoscope; Karl Storz). In autofluorescence, substances under the mucous membrane are stimulated by light of a specific wavelength and are temporarily excited to a higher energy level. When they subsequently return to their ground state, this energy is released again in the form of light at a different wavelength from that used for stimulation. Cancerous lesions display loss of autofluorescence due to malignant changes in epithelium and subepithelial stroma. Autofluorescence may be suitable for highlighting the tumor site and other areas of interest, in particular the margins of the lesion, in a more unspecific way. Such mucosal areas can then be analyzed on a microscopic level by confocal endomicroscopy in a complementary fashion. In our protocol, the margins of potential lesions displayed by autofluorescence were marked with toluidine blue and were photographically documented (**Figure 2**).

For confocal imaging, the rigid probe was placed in direct contact with the center of tumor and the highlighted margins following the intravenous administration of the contrast agent fluorescein (5 mL of 10% solution; Alcon Pharma). Administration of fluorescein has been used and well evaluated before by our group³⁻⁹ during extensive investigations in the GI tract. Use of systemic fluorescein allows detailed visualization of vasculature and respective pathologic angiogenic effects of malignant growth.¹³

Confocal imaging started immediately after application of the contrast agent in the toluidine blue and focused on the marked tumor margins and the center of the tumor. In addition to the standardized *in vivo* imaging, resected specimens were also investigated *ex vivo*. The respective biopsy specimens were positioned on the tip of the rigid confocal laser endoscope. We then topically applied 0.02% acriflavine solution in saline (Sigma Pharmaceuticals). Acriflavine stains subcellular details and nuclei and therefore adds important information to the fluorescein-based analysis.

In our study the surgeon was asked to interpret the confocal images in real time during standard endoscopy without any preselection of the imaging data. Afterwards, sampled images were reanalyzed and related to the well-established gold standard, histologic analysis. The respective biopsy specimens were taken precisely at each spot that had been endoscopically evaluated. Crude imaging data from each patient were correlated with traditional histopathologic findings in interdisciplinary conferences (surgeon and/or confocal investigator, pathologist).

RESULTS

Confocal laser endomicroscopy was performed with the rigid prototype probe during standard procedures in 15 patients with SCCHN. Fluorescein was used as a contrast agent. Confocal imaging provided instant real-time microscopic imaging in SCCHN during the ongoing surgical procedure in all patients. Rigid confocal endoscopy was safe. No adverse effects from intrave-

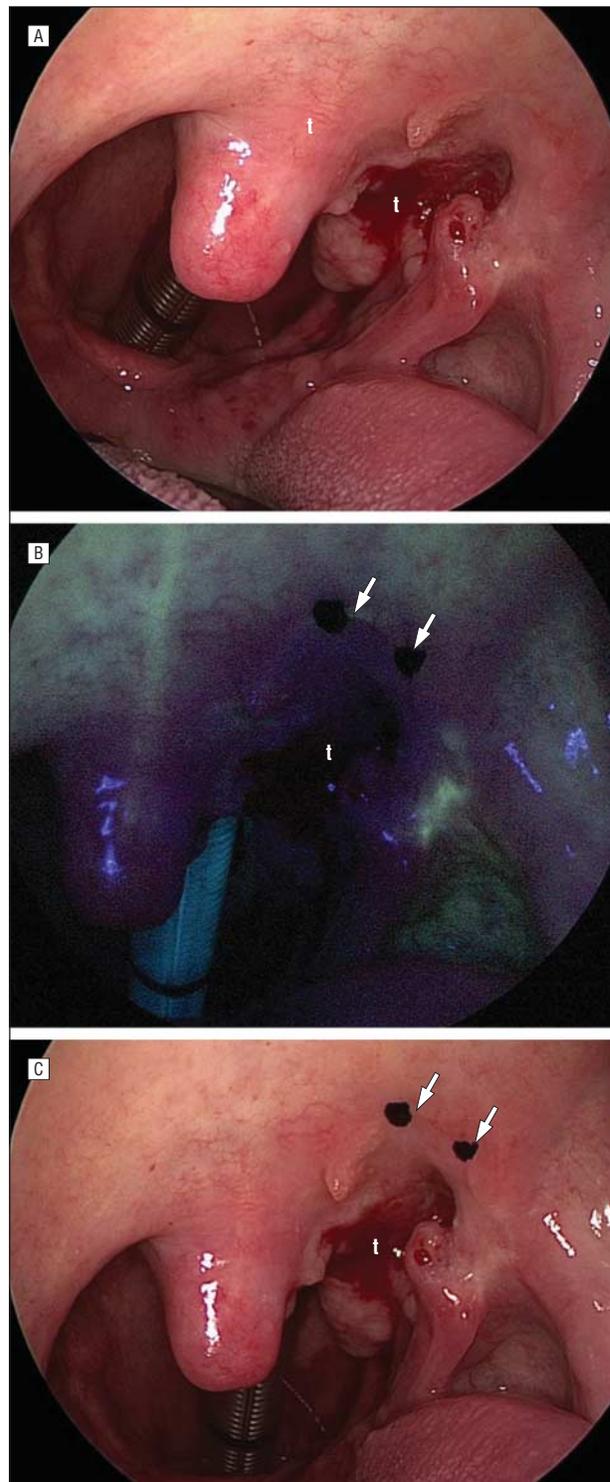


Figure 2. Intraoperative images of oropharyngeal carcinoma tumor (t). A, Autofluorescence procedure. B, Tumor margins are marked with color spots (arrows) to define the exact extent of the lesion. C, Postprocedural autofluorescence examination, with arrows indicating the marked margins.

nous application of fluorescein occurred during or after our investigations.

In total, 2078 images from 15 patients were digitally stored (mean, 138.5 images per patient). Imaging of mucosal lesions of almost every anatomic region in the head and neck *in vivo* was readily feasible after injection of fluo-

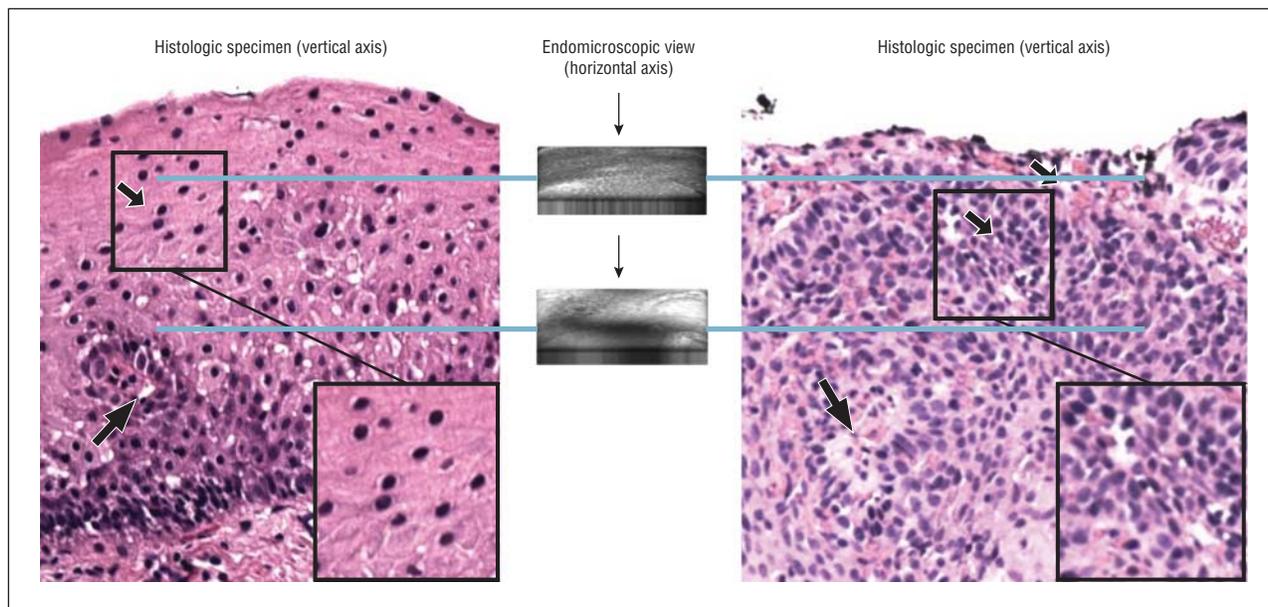


Figure 3. Conventional histologic images (hematoxylin-eosin) of hypopharyngeal healthy mucosa (left) vs squamous cell carcinoma (right) in standard vertical axes and endomicroscopic view (middle) highlighting classic changes of cell patterns and architecture in invasive SCC.

rescein using the rigid confocal endomicroscope. Most images were of acceptable or good quality. Movement artifacts caused by respiration during general anesthesia or probe instability were the most common artifacts, followed by mucus or blood (after biopsy) adherent to the confocal window. While manipulation of the flexible endoscope in the oral cavity was limited, handling of the prototype rigid confocal laser endoscope was much more convenient for the examiner. Despite the patient's respiration movements and probe instability, the rigid endoscope contacted the appropriate standard Kleinsasser's or Weerda's type direct laryngoscope during the procedure, and gentle pressure against the region of interest helped to stabilize the probe. On average, rigid confocal imaging added 10 to 15 minutes to the total examination time.

As expected, imaging without contrast agent did not provide sufficient information because no cellular or subcellular structures could be identified. This was the case in both normal mucosa and in invasive carcinoma of different regions. According to the results in flexible confocal imaging, after intravenous administration of fluorescein, a differentiation of the matrix of the connective tissue of the epithelial layer was possible. Conventional histologic analysis enabled differentiation of the mucosal and submucosal layer on a vertical axis, whereas endomicroscopy provided images in a horizontal axis. Previously described typical aspects of normal mucosa such as the thick epithelium of the buccal area and the tongue and the thinner epithelium of the floor of the mouth were displayed at high resolution.

We reproducibly demonstrated the following conventional histologic characteristics within the imaging data of normal mucosa in different locations of the head and neck (**Figures 3, 4, and 5**):

- Architecture of tissue
 - Homogeneous configuration of the superficial layers
- Characteristics of cells
 - Regular and homogeneous nuclear structure

- Clearly defined cellular structure
- Characteristics of capillaries
 - One to 2 capillaries per field of view
 - Regular and longitudinal configuration

The homogeneous configuration of superficial squamous epithelial layers and capillaries with erythrocytes in the deeper layers of the mucosa were precisely and reproducibly visualized (Figure 4).

Acridflavine was used as a topical dye, which particularly highlighted nuclei and cell membranes. In the ex vivo specimens, staining of the mucosa with acridflavine resulted in strong fluorescence of the nuclei of the surface epithelium. Close examination of our images showed variations in the fluorescent staining intensity within the nuclei, demonstrating subcellular resolution of the endomicroscope. Normal mucosa showed regular and constant configuration of cell nuclei (Figure 5).

In addition, we were able to reproducibly apply the following descriptive criteria of SCC within the confocally generated imaging data and compare with conventional histologic findings:

- Characteristics of capillaries
 - One to 2 capillaries per field of view
- Architecture of tissue
 - Nonhomogeneous configuration of the superficial layers
- Characteristics of cells
 - Irregular cellular and nuclear structure
 - Blurry cellular definition
- Characteristics of capillaries
 - Three to 4 capillaries per field of view
 - Extended and irregular configuration.

For diagnostic application of confocal endomicroscopy with the rigid endoscope, we also used intravenous fluorescein as a contrast agent. In keeping with our previous experience using the flexible instrument, we also

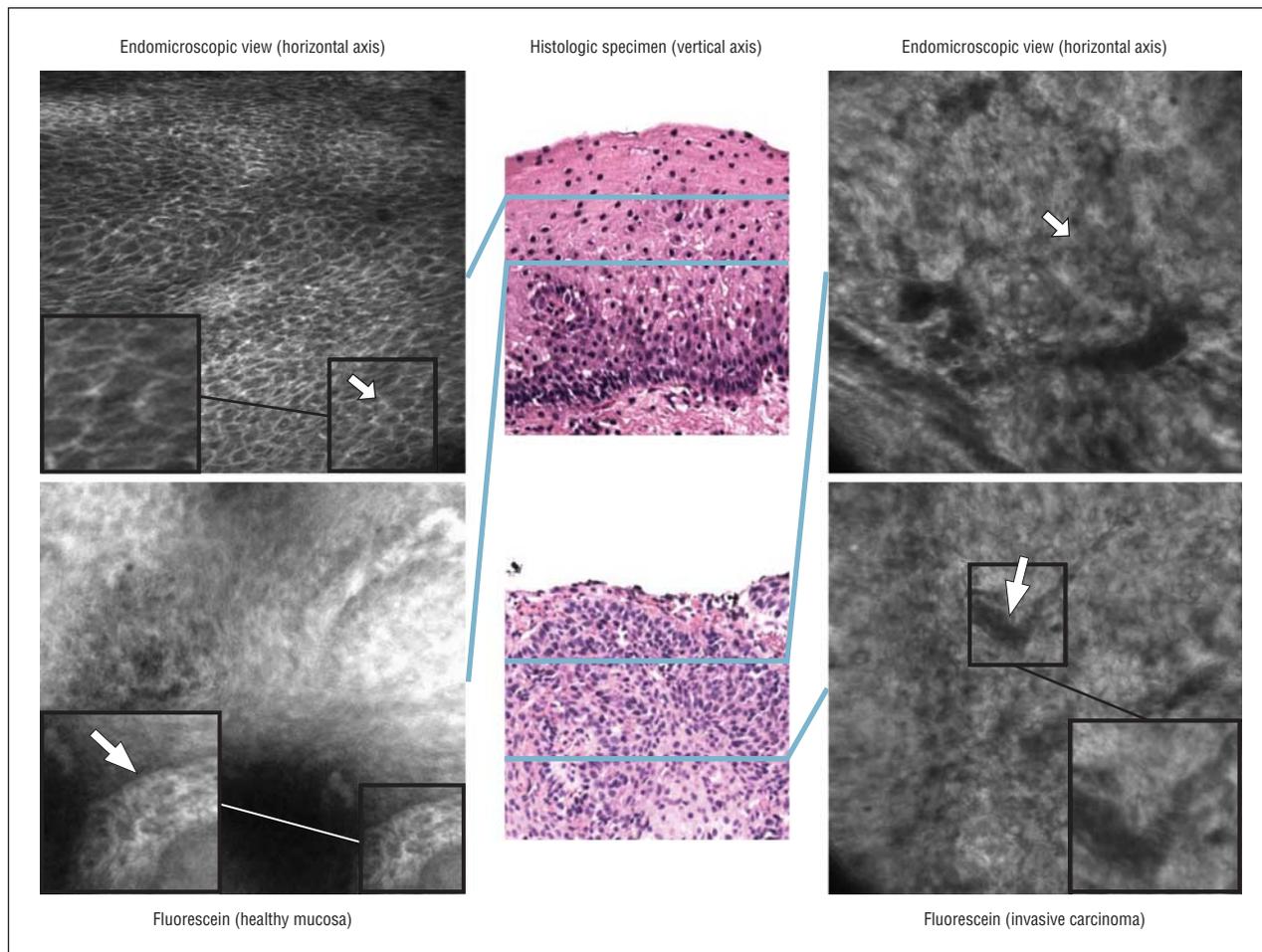


Figure 4. Confocal imaging (left and right) and histologic views (middle) after intravenous fluorescein sodium administration. In contrast to healthy mucosa (left) with homogeneous superficial layers (top arrow) and capillaries with erythrocytes in deeper layers (bottom arrow), all layers of squamous cell carcinoma (right) show irregular cell patterns and architecture (top arrow) and extended capillaries and neoangiogenesis (bottom arrow).

identified typical morphologic signs of malignancy. The confocal images showed extended capillaries and neoangiogenesis, which correlated well with the histologic findings from the same specimen. Neoplastic lesions also showed irregular cellular architecture (Figure 4). In a number of cases, we were able to detect the infiltration depth by serial imaging of various cell layers.

In addition to using intravenous fluorescein, we also used topical acriflavine *ex vivo* in SCC of the hypopharynx. Confocal endomicroscopy with the rigid probe revealed irregular tissue architecture. The images also showed different sizes of nuclei and leaking of contrast agent into the intracellular space (Figure 5).

A disadvantage was that after topical application of acriflavine, the imaging depth was limited to the superficial 50 μm of the mucosa. In addition, the positioning of the *ex vivo* biopsy specimens on the tip of the rigid laser probe required an individual learning curve on the part of the investigator.

COMMENT

This is the first study to our knowledge to evaluate the intraoperative application of confocal endomicroscopy

with a prototype of a novel rigid laser endoscope in SCCHN. In a pilot study, our group¹⁰ had demonstrated the feasibility of *in vivo* confocal endomicroscopy after intravenous fluorescein staining and *ex vivo* topical application of acriflavine. For that investigation, examinations were performed with a flexible confocal endoscope, well known from a range of proven applications in the field of gastroenterology.³⁻⁹ The application of that flexible instrument was limited to different regions of the oropharynx and the oral cavity.

With the new prototype of a now rigid endomicroscope used for the present study, we were able to test the innovative technique in less accessible locations, such as SCC of the larynx and the hypopharynx, in a fashion resembling the rigid endoscopic procedures as they are typically conducted in the field of otolaryngology. Demands for sterility were met by implementing a removable sterilizable rigid outer sheath design.

The use of 2 fluorescent contrast agents proved to be feasible, both of which are used elsewhere in clinical medicine. Acriflavine is a commonly used topical antiseptic agent in wound healing. Fluorescein is commonly used in ophthalmology as an indicator dye for imaging of retinal microvascular damage associated with diabetes. Both

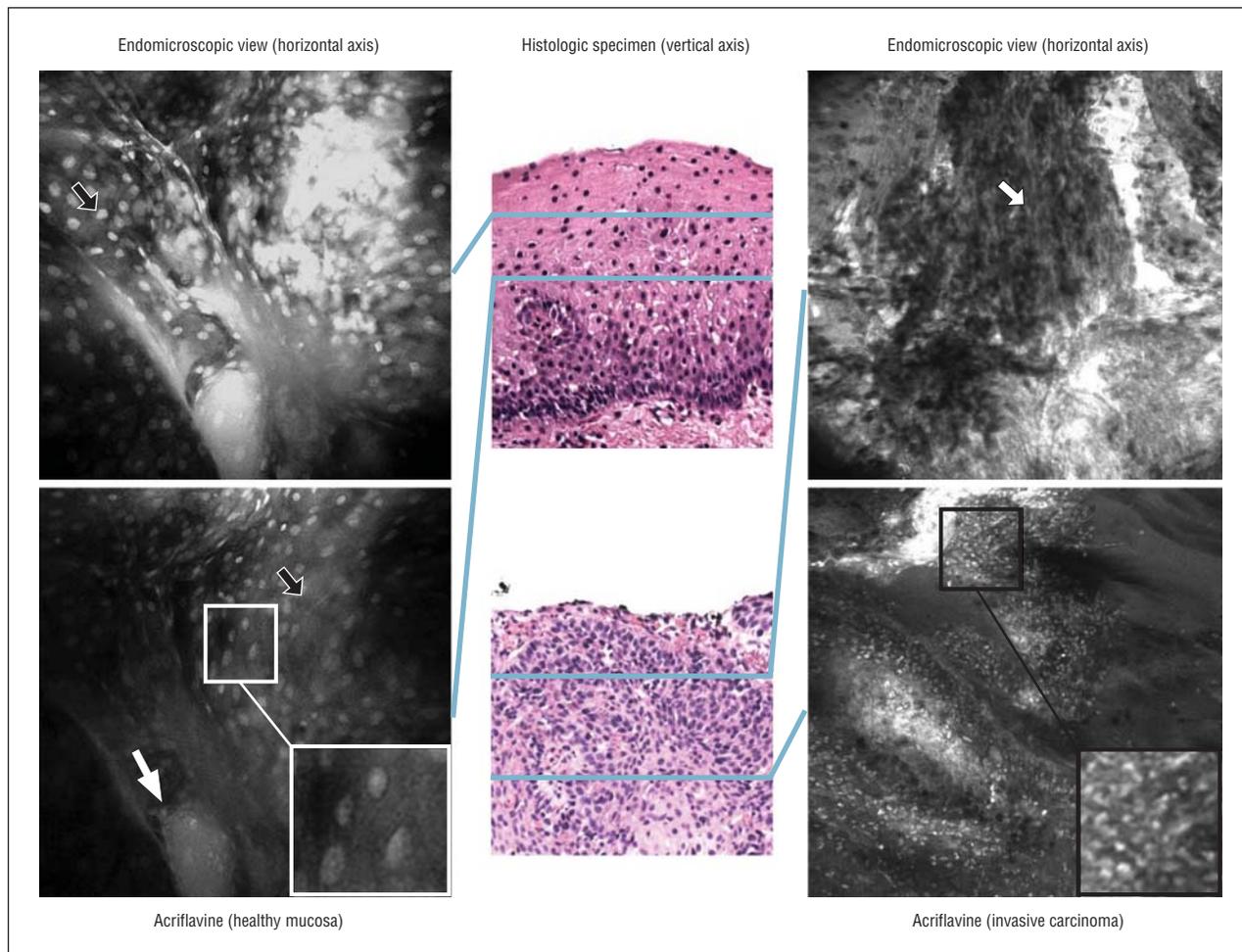


Figure 5. Confocal imaging after topical acriflavine hydrochloride application in ex vivo specimens of healthy mucosa (left) and squamous cell carcinoma (SCC) (right); histologic specimens are also shown (middle). The healthy mucosa demonstrates regular configuration of cell nuclei (black arrows) and membranes as well as capillaries in deeper layers (white arrow). The SCC, in contrast, shows polymorphic nuclei, irregular tissue architecture in all layers.

agents have a long and a substantial history of clinical use. The most common adverse effects associated with the administration of fluorescein (nausea and vomiting) are transient and minor. More severe adverse effects, such as a vasovagal response or cardiac or respiratory effects, are extremely rare.¹⁴ Structures that can be visualized with fluorescein include blood vessels, red blood cells, surface epithelial cells, and the connective tissue matrix of the lamina propria. Cell nuclei were only rarely visualized after the administration of fluorescein because fluorescein only occasionally crosses the lipid membranes and does not bind to nucleic acids. No adverse effects due to intravenous application of fluorescein occurred during or after our investigations. This is in keeping with the extensive experience using this systemic fluorescent dye for confocal endomicroscopy in gastroenterology.¹³⁻¹⁵

Acriflavine, in contrast, crosses cell membranes and displays a strong specificity for labeling acidic constituents. It also demonstrates some staining specificity for collagen and elastin. Unlike fluorescein, topical acriflavine does not result in staining of the deeper layers of the mucosa. Topical acriflavine predominantly stains the nuclei of the surface epithelium.⁴

Our unique protocol offers a choice of agents and thus the option to interpret their respective indicators, which opens the potential to obtain different kinds of microscopic information from the tissue. In the absence of safety data on the use of acriflavine in vivo, we used it as a topical dye ex vivo to acquire data complementary to that provided by systemic fluorescein. Given the encouraging data gathered from these ex vivo samples, it will be a goal of ongoing investigations to identify a comparable topical staining solution that is suitable for in vivo application.

As we could with the flexible instrument, we were able to use the rigid probe to identify and describe typical morphologic signs of malignancy and dysplasia like irregular cell patterns and increased vascular support after intravenous application of fluorescein. Furthermore, the confocal images showed extended capillarization and neovascularization, which correlated well with the histologic findings of the same specimen. Neoplastic lesions also showed irregular architecture and unclear cell borders. In addition, after topical application of acriflavine ex vivo, confocal endomicroscopy with the rigid probe revealed an irregular architecture of the tissue in all anatomical regions

with SCC. The obtained images displayed polymorphic nuclei and leaking of contrast agent.

However, the positioning of the ex vivo probes on the tip of the rigid instrument occasionally turned out to be quite difficult. In particular the position of the probe in the right axis (superficial cell layers in horizontal axis) with contact to the instrument was important to achieve reproducible information from the different tissue layers. Because the imaging depth was limited to the top 250 μm of the mucosa, evaluation of potential further infiltration and identification of submucosal spread with confocal imaging were limited.

Overall, most images were of acceptable or good quality. Movement of the tip of the probe, triggered by probe instability during confocal imaging, caused the most common artifact. While manipulation of the flexible endoscope was limited to the most easily accessible anatomic regions of the oral cavity, manipulation of the prototype rigid confocal laser endoscope allowed visualizations even within the larynx and the hypopharynx and was much more convenient for the examiner. Despite the patient's respiration movements and probe instability, the rigid endoscope contacted the appropriate standard Kleinsasser's or Weerda's type direct laryngoscope during the procedure, and gentle pressure against the region of interest helped to stabilize the probe.

For the first time, we are now able to describe criteria for healthy mucosa and malignant changes by using this method in SCCHN. Considering the impact of a virtual real-time histologic analysis, we believe that this technology might be considered a very promising development. It may carry potential for quicker intraoperative diagnosis, less need for multiple frozen sections, and more precise resection of margins. Especially in the field of otolaryngology-head and neck surgery, malignant and premalignant lesions are frequently associated with submucosal spread and/or field cancerization, sometimes secondary to long-term exposure to tobacco smoke and alcohol. In such cases there would be a substantial benefit in a noninvasive diagnostic tool, especially in functionally sensitive regions such as the larynx. In our study we also applied the new prototype of a laser endomicroscope in combination with the already available technology of autofluorescence. This may add to the potential of the method because autofluorescence is suitable for highlighting areas of interest in a more non-specific way. Such mucosal areas can then be analyzed on a microscopic level by endomicroscopy in a complementary fashion.

Our descriptive findings of abnormalities in tissues with dysplasia or SCC vs healthy mucosa within the head and neck appear suitable for translation into a more objective and rater-independent algorithm of analysis. This step was taken before implementing this technology in the diagnostic workup of gastric and intestinal lesions.³⁻⁹ Therefore, it is a prominent goal of ongoing investigations in this field to develop and validate a user-independent scoring system representing the typical cellular and subcellular findings of malignant growth in squamous cell epithelia. Furthermore, beyond soft-tissue evaluation, our innovative technique might prove valuable in visualiz-

ing the bony or cartilaginous structures that often represent the deep resection margins of a tumor, especially in the head and neck. The present study demonstrated that biopsy specimens of bony structures could be visualized by subsurface scans with confocal laser scanning microscopy. Therefore, real-time histologic confirmation of bony and/or cartilaginous margins in vivo during endoscopy after resection of a lesion might be an additional topic for investigation in the head and neck region.¹⁶

In summary, confocal endomicroscopy is now available as an innovative tool for the diagnostic and therapeutic evaluation of SCCHN. The use of the prototype rigid endomicroscope is feasible and safe, and it permitted the identification and description of typical microarchitectural aspects of SCCHN. Although more prospective studies are warranted to further elucidate the clinical value of this technology, the present results using confocal imaging with rigid endomicroscopy are encouraging. This method carries high potential for dynamic functional in vivo imaging in head and neck cancer. It holds the promise of predicting aspects of malignancy or dysplasia and of targeting biopsies and resection margins in this functionally sensitive anatomic area.

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Author Contributions: Drs Pogorzelski and Gosepath had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Pogorzelski, Goetz, Kiesslich, and Gosepath. *Acquisition of data:* Pogorzelski, Hanenkamp, Goetz, and Gosepath. *Analysis and interpretation of data:* Pogorzelski, Hanenkamp, Goetz, Kiesslich, and Gosepath. *Drafting of the manuscript:* Pogorzelski. *Critical revision of the manuscript for important intellectual content:* Pogorzelski, Hanenkamp, Goetz, Kiesslich, and Gosepath. *Obtained funding:* Pogorzelski, Hanenkamp, Kiesslich, and Gosepath. *Administrative, technical, and material support:* Hanenkamp, Goetz, and Kiesslich. *Study supervision:* Goetz, Kiesslich, and Gosepath.

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