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Autofluorescence Imaging and Spectroscopy of Normal and Malignant Mucosa in Patients with Head and Neck Cancer

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Background and Objective: **An early detection of oral cancer might improve the patient's prognosis. We present preliminary results of autofluorescence photodetection of cancerous oral mucosa.**
Materials and Methods: **49 patients were investigated altogether. In 30 patients, malignant and healthy oral mucosa were excited with violet light (λ = 375 to 440 nm). Images were recorded by a sensitive CCD camera. Spectrophotometric analysis in the green spectral range was performed on tumorous and innocuous mucosa in 36 patients.**
Results: **In 13 patients (43.3%), tumors were subjectively better distinguishable from their surroundings through a reduction of green autofluorescence than by ordinary inspection. Tumor detection abilities varied for different locations and tumor morphologies. Spectral analysis showed contrasts in autofluorescence intensities between tumor and normal tissues in 34 patients (94.4%). Autofluorescence spectra of normal mucosa varied both inter- and intraindividually.**
Conclusions: **Using violet excitation light, camera-based autofluorescence photodetection in the green spectral range presented a highly promising tool for the diagnosis of oral malignomas in almost half of the cases examined. The possible ways on how the obtained results could serve to find a more advanced method for a precise tumor detection in the oral cavity are being discussed. Lasers Surg. Med. 25:323–334, 1999.**
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Key words: autofluorescence photodetection; native cellular fluorescence; NCF; oral cancer; spectroscopy; squamous cell carcinoma

INTRODUCTION

Carcinomas of the upper aerodigestive tract present a big problem in modern health care. In the United States, cancer of the oral cavity and pharynx is the sixth most frequently diagnosed cancer in white males, and even ranks fourth among black males [1]. Nearly 30,000 cases of oral cancer were estimated for the United States in 1994, and almost 8000 people died of this disease [2]. From 1974 until 1990, five-year relative survival rates for cancer of the oral cavity and pharynx have remained stable at 55% [1], indicating that no major progress in therapy has been achieved within the last two decades.

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Within the German population, morbidity- and mortality rates of squamous cell carcinoma of the oral cavity or oropharynx respectively show an upward tendency. For the time period from 1978 to 1993, the estimated incidence of malignant lesions of the oral cavity (ICD 141, ICD 143–145) within the male German population has risen from 8.4 up to 15.1, thus showing a factor two increase. In the same period of time, the estimated mortality of this disease rose from 2.7 to 7.0 per 100,000 male Germans [3].

Yet, early detection followed by radical surgery could highly increase the cure rates of oral cancer, as published by Silverman et al. [4]. Of 1467 patients with oral cancer investigated, 3-year survival rates dramatically increased from 39% to 78% for patients with lesions less than 4 cm in diameter without evidence of spreading to regional lymph nodes. However, especially early lesions are often hard to detect and are sometimes overlooked even by experienced clinicians with a high diagnostic acumen. These early carcinomas might appear as flat, inconspicuous irregularities of the mucosal surface and lack typical morphological characteristics of malignant tumors. Especially susceptible are patients suffering from a primary oropharyngeal tumor, as studies by Shibuya et al. have shown that there is an increased probability for them to develop a secondary carcinoma [5]. Of 1429 tumor-patients evaluated, multiple secondary carcinomas occurred in 117. In 64% of these patients the tumors were localized in the upper aerodigestive tract. Long-term damage of the oral mucosa due to exogenous noxa ("condemned mucosa") might lead to this so called "field cancerization", as first proposed by Slaughter et al. in 1953 [6].

To ameliorate diagnosis of early or secondary malignant transformations of oral and pharyngeal mucosa, several different screening methods for "high risk patients" have been developed and discussed by numerous international research groups over the last decades.

First staining experiments of malignant oral mucosa with Lugol's iodine were performed by Morgenroth in 1957 [7], using a technique originally developed for the detection of cervical neoplasms by Schiller [8]. The iodine stains by reacting with glycogen, which is supposed to be present in lower concentrations within neoplastic tissue compared to healthy stratified epithelium. However, he did not obtain any reliable results, as intracellular glycogen levels didn't seem to follow any common pattern.

In 1963, Richart presented first experiments with toluidine blue in the field of gynecology [9]. Toluidine blue is a metachromatic dye of the thiazine group that binds to intracellular DNA and RNA. Selective staining properties of toluidine blue for neoplastic tissue might result from either higher levels of nucleic acids within neoplastic cells or wider and more numerous intracellular canals, which enhance penetration of the dye into the tissue [10]. Using this stain, he was able to accurately outline the surface margins of cervical carcinomas in situ. In the following decade, toluidine blue was subject to numerous trials and was controversially discussed in the field of oto-rhino-laryngology [11–14]. Generally this diagnostic method seems to provide the physician with considerable assistance in both detection and delineation of precancerous and cancerous lesions of the oral cavity. But even though most promising results have been reported, general weaknesses of the method—most apparent in a study presented by Sabes et al. [14], who found a high percentage of false-positive and false-negative results in the tumors they examined—seem to have prevented it from becoming more widely used.

Since the early 1940s, researcher's attention has been drawn to a selective intracellular deposition of fluorescent markers. Several groups have reported on the affinity of porphyrins to malignant tissue and the possibility of its exploitation for an enhanced diagnosis of malignant neoplasms [15–18]. Leonard et al. tested the feasibility of intravenously applied hematoporphyrin derivative for the diagnosis of neoplasias of the oral cavity, the pharynx, hypopharynx and larynx [19]. They found this method to be highly reliable for the detection of mucosal carcinomas, as they observed a characteristic red fluorescence in all 29 lesions investigated. Major drawbacks are the inability to clearly delineate the tumor borders and a general photosensitation of the whole skin for several days following application of hematoporphyrin derivative. In 1972, Dunn et al. investigated the effectiveness of tetracycline-induced fluorescence in 54 patients with cancer of the upper digestive tract [20]. The results he obtained didn't justify the tremendous amount of time and the large technical equipment necessary for each examination.

Another recently introduced fluorescent marker with tumor localizing properties is 5-aminolevulinic acid induced protoporphyrin IX (PPIX). Topical or systemic administration of 5-aminolevulinic acid (5-ALA) results in a selec-

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tive accumulation of PPIX in neoplastic tissue, which is most possibly due to altered activity levels of the enzymes of the heme biosynthetic pathway within transformed cells [21–24]. Kennedy et al. were the first to propose 5-ALA induced PPIX for the detection of pre-malignant and malignant oral mucosa [25]. In previous publications [26–28], our group has reported on the successful use of topically applied 5-ALA for a fluorescence-aided staining of oral squamous cell carcinomas. To us, this method represents a very useful tool in demarcation and detection of both early and advanced oral cancer, and is at the same time reproducible and easily conductible without any side effects for the patients. For further evaluation, both specificity and sensitivity are currently being tested on a larger number of patients.

Finally, several groups have performed autofluorescence measurements of healthy and malignant tissue in order to determine its use for the detection of tumors. Policard is considered the first to have recognized the presence of endogenous porphyrins in human tumors in 1924 [29]. 30 years later, in the year 1954, it has been observed by Ronchese that ulceration is essential for the production of red fluorescence on human cutaneous squamous cell carcinoma [30]. In the early 1960s, Ghadially et al. found a bright red fluorescence limited to the exterior of ulcerated tumors, which he identified to be a result of microbial porphyrin synthesis that can be wiped off the surface [31–33]. He concluded that, in the presence of 5-ALA, many bacterial organisms that inhabit necrotic tissue are able to produce red fluorescing porphyrins. Even though researchers have extended their investigations of endogenous porphyrins in tumors to the oral cavity [34–36], no routine diagnostic method on its basis has been developed so far.

Apart from porphyrins, several naturally occurring tissue components are held responsible for producing the so-called NCF (native cellular fluorescence); each fluorophore's contribution to the overall fluorescence emission depending on the excitation wavelengths. The relevant fluorochromes are mainly localized in the submucosa. Alfano et al. [37] was the first to observe different spectral profiles of normal and cancerous tissues. Those alterations in both autofluorescence characteristics and intensities of neoplastic lesions and healthy mucosa can either be due to a variation in the concentrations of the fluorescing components mentioned above or to changed morphological tissue structures. Thus, a thickening of the epithelium or overlying tumor tissue respectively may cause a decrease of the autofluorescence signals from the deeper layers of tissue. In 1995, four different fluorescence scans (using varying excitation- and emission wavelengths) were conducted by Kolli and co-workers on 31 patients with mucosal neoplasms of the oral cavity [38]. They concluded that in vivo NCF properties of oral cancer differ from those of normal upper aerodigestive epithelia.

As for the clinical use, several research groups have tried to evaluate the practicability of these theoretical findings to develop reliable imaging techniques for the detection of oral cancer. In 1992, Kluftinger et al. reported on the "Detection of squamous cell cancer and pre-cancerous lesions by imaging of tissue autofluorescence in the hamster cheek pouch model" [39]. They formed pseudocolor images from the ratio of red to green fluorescence (R/G Ratio)—ratios greater than 1.5 were defined as malignant or premalignant. Using this method, they determined a sensitivity of 100% and a specificity of 80% for 32 separately examined sites. They attribute the high ratios found in neoplastic lesions to a selective porphyrin accumulation within the tumors and a decreased green autofluorescence. Using the LIFE system (Xillix Technologies Corp., Vancouver, Canada) which was originally designed for an application in the lung, Lloyd-Harries et al. found that the autofluorescence characteristics of healthy and malignant laryngeal mucosa closely resemble those of the bronchial wall. Therefore, he concluded, the LIFE system could also be used for the detection of neoplasms in the larynx [40]. Fryen et al. performed both fluorescence microscopy and spectroscopic examinations on 89 biopsies (32 cancer specimens of the upper aerodigestive tract (UADT) and 57 specimens of normal oral mucosa) at an excitation wavelength of 365 nm [41]. In contrast to results in the literature, they could not prove a homogeneous fluorescence gradient between marginal epithelium and the tumor itself. However, tumor borders were clearly delineated by an increase of autofluorescence intensities.

This investigation aims to evaluate the value of autofluorescence photodetection of oral neoplasms in the green spectral range utilizing a band of excitation wavelengths in the near UV to blue (375–440 nm). High resolution images and spectral data analysis from neoplastic- and surrounding host tissue are presented and discussed. Spectrophotometric examination of non-malignant

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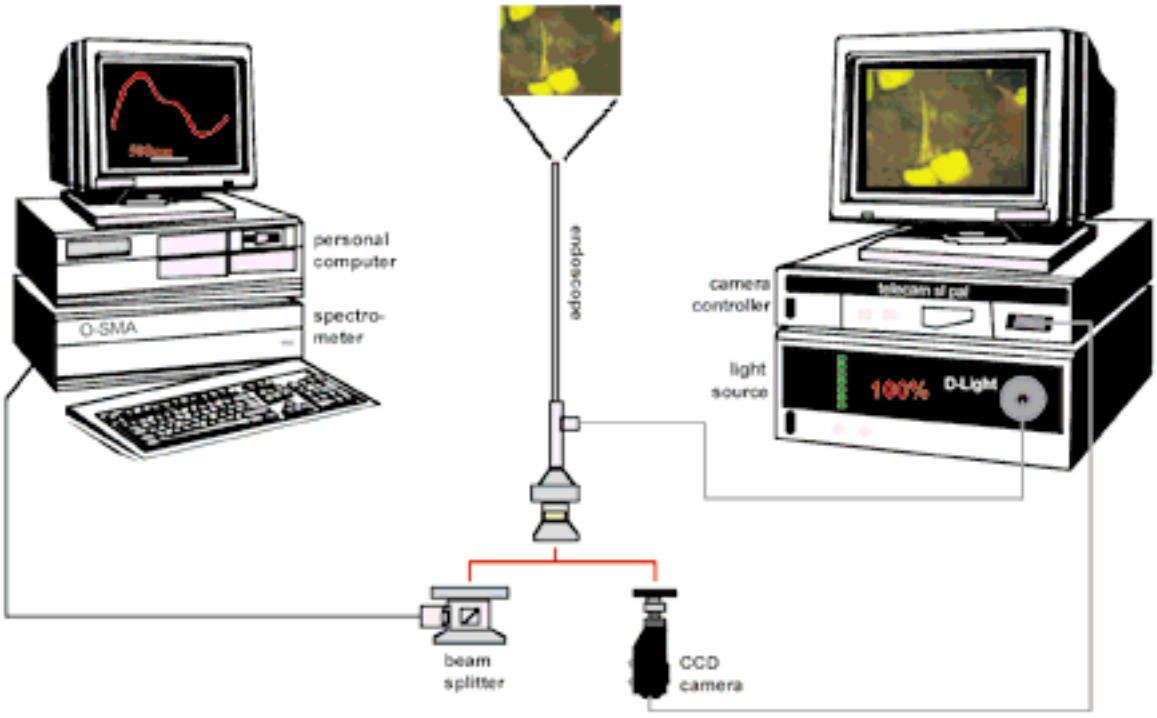

Fig. 1. Experimental setup used for autofluorescence measurements of normal and malignant oral mucosa.

mucosa serve to quantify inter- and intraindividual variations of autofluorescence intensities at several anatomical locations of the oral cavity.

MATERIALS AND METHODS

Patients

This investigation included one healthy volunteer (age 23) and 49 patients (mean age 57.6 a, Std. Dev. 8.7, range 39 to 81 a) bearing a histologically proven squamous cell carcinoma of the oral cavity or the oropharynx, respectively. An informed consent was obtained from all patients who underwent examination.

In vivo fluorescence imaging of the oral squamous cell carcinomas was performed on 30 out of a total of 49 patients (mean age 58.8 a, Std. Dev. 8.8, range 42 to 81 a). For each patient, fluorescence and white light images were recorded in vivo from various sites of the oral cavity and the oropharynx.

Spectrophotometric measurements were made on the healthy person and 36 out of a total of 49 patients (mean age 57.3 a, Std. Dev. 9.4, range 39 to 81 a). Spectral data were generated from each tumor and surrounding host tissue as well as from multiple standard screening points (hard palate, buccal mucosa, dorsum of tongue, inner lip). From the healthy volunteer, multiple autofluorescence spectra were taken from all anatomical areas of the oral cavity (hard palate, buccal mucosa, dorsum of tongue, inner lip, floor of the mouth, rim of tongue, alveolar ridge) and parts of the oropharynx (soft palate), following a strict pattern of squares.

Experimental Setup

The experimental setup for the measurements is presented in Figure 1.

In vivo picture generation. In vivo tissue excitation was carried out by a modified Xenon short arc lamp (D-Light, Art. Nr. 20133201, Storz, Tuttlingen, Germany) at wavelengths of 375 to 440 nm. A footswitch allowed changing between common white light illumination and fluorescence excitation. For both illumination and detection of the examined sites in the oral cavity, an endoscope was slightly modified (0°, Art. Nr. 7200 A, Storz, Tuttlingen, Germany). To maximize excitation intensities, the illumination fiber bundle had no connectors and directly fit into the light source. As a further alteration to a standard endoscope, the applied device could be optionally equipped with a blocking filter (OG515, Schott, Mainz, Germany), allowing transmission of wavelengths above 515 nm (100% transmission at wavelengths >525 nm). This leads to a total blocking of the remitted excitation light. A highly sensitive color

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CCD (charge coupled device) camera (Telecam SL PAL, Art. Nr. 20212020, Storz, Tuttlingen, Germany) with target integration over several video frames up to 2 s in combination with a video recorder (Video-Recorder S-VHS, Art. Nr. AG-7350, Panasonic, Osaka, Japan) were used for acquisition and recording of high quality pictures of whitelight- and fluorescence findings.

Spectral analysis. Optical spectra were taken of each patient from several sites of the oral cavity. All spectral data were collected in a darkened room to prevent ambient light from influencing the results. For a reproducible and comparable recording of each spectrum, the measurements were made in a fixed geometrical setup using a resection loop protruding exactly 12 mm from the front end of the endoscope. All spectra were taken from a spot in the center of the fluorescence image with about 2 mm in diameter. A beam splitter (70/30) fixed to the eyepiece of the endoscope allowed for 70% of the remitted fluorescence light to be collected by a fused silica fiber (HNC 600, core diameter 600 μm, Laser Components, Gilching, Germany) which was connected to an optical multichannel analyzer (O-SMA, SI Instruments, Gilching, Germany). The remaining light (30%) was transmitted to the examining clinician to ensure a correct and perpendicular positioning of the distal tip of the resection loop onto the tissue.

An adequate blocking of the remitted excitation light was achieved by a combination of two longpass filters (KV430, GG455, Schott, Mainz, Germany) at the entrance of the spectrometer. No additional blocking filter was used on the endoscope for these measurements. Spectra were recorded from 450 to 750 nm. Each spectrum automatically underwent digital background subtraction and a correction compensating for the wavelength dependent sensitivity of the system.

Final spectral analysis at wavelengths representing tissue autofluorescence (470–600 nm) was performed on a personal computer using scientific graphing software (SigmaPlot 4.0, Jandel Scientific, San Rafael, California, USA).

RESULTS

Fluorescence Imaging

Due to the filter combination, no blue excitation light was picked up by the camera, so only genuine green tissue autofluorescence was recorded by the camera and displayed on the screen. Both normal and cancerous oral mucosa appeared in a green color on the acquired images. However, neoplastic tissue showed lower autofluorescence intensities in general, so malignant lesions were demarcated from the adjacent normal tissue by a darker shade of green (Fig. 2).

In 13 of 30 patients investigated (43.3%), subjective demarcation properties of autofluorescence photodetection were classified as good or very good respectively; i.e. dark parts on the images correlated well with the lesions found under common white light illumination and the tumor borders had sharp outlines on the autofluorescence pictures. 23.3% (7 of 30 patients) showed sufficient results. The areas of tumors were noticeably darker than surrounding healthy tissue, but did not show sharp contrasts at the edges of the carcinomas. In the remaining 10 patients, the malignant tumors were not at all distinguishable from their host tissues by means of autofluorescence photodetection (Table 1).

Flat epithelial lesions—even when widespread—showed greater contrasts in autofluorescence intensities as compared to exophytic tumors. Moreover, demarcation abilities of autofluorescence photodetection of oral carcinomas varied for different locations in the oral cavity. For example, squamous cell carcinomas of the rim of tongue were more difficult to differentiate from innocuous tissue as neoplastic lesions from the floor of the mouth or the hard palate (Table 1).

In normal oral mucosa, highest autofluorescence intensities were evident at the hard palate. The back of the tongue showed a bright red fluorescence typical for endogenous porphyrins, restricted to the area covered by lingual papillae. A similar reddish fluorescence signal was noticed originating from bacterial coating of tumors or tartar respectively. In only 10 of 30 tumors investigated (33.3%), most of the malignant lesion's surfaces disposed of a strong red fluorescence on the acquired images. However, the distribution of red fluorescing areas on the tumorous lesions was varying in size and appearance and didn't seem to follow any common pattern and is therefore not suitable for a precise tumor demarcation.

Spectral Data Analysis

Averaged spectra from both tumorous lesions and adjacent tissues peaked at 511 nm (Fig. 3). In 34 of 36 patients investigated (94.4%), autofluorescence spectra of neoplastic tissue disposed of lower overall intensities and showed lower maximum values at 511 nm compared to

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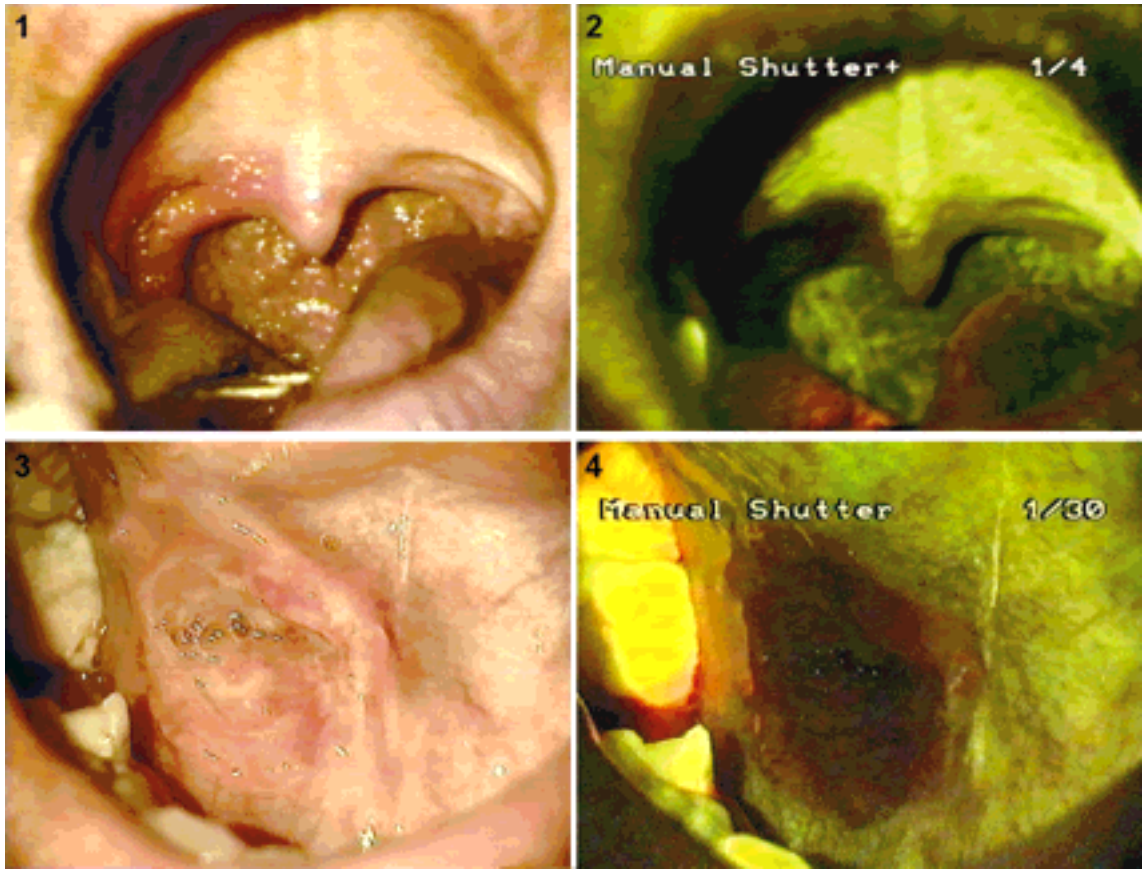

Fig. 2. Illustration of macroscopically visible contrasts in autofluorescence between tumor- and healthy tissues. **Panel 1** shows a carcinoma of the right tonsillar sinus in a 57 year old patient under white light illumination. **Panel 2** reveals the autofluorescence seen under excitation with blue-violet light at the same localization. **Panels 3, 4** picture a squamous cell carcinoma of the right floor of the mouth in a 45 year old patient. Panel 3 shows the malignant lesion and its surrounding tissue under ordinary white light illumination, while Panel 4 depicts the autofluorescence findings of the same tumor under fluorescence excitation.

TABLE 1. Subjective Demarcation Properties of Autofluorescence Photodetection of Oral Squamous Cell Carcinoma

Subjective demarcation of neoplasms by means of autofluorescence photodetection	Number of patients or tumors	Floor of the mouth	Hard plate	Rim of tongue	Tonsillar sinus	Soft palate	Oropharynx
Very good	2	1	0	0	1	0	0
Good	11	2	3	0	3	2	1
Sufficient	7	3	0	2	1	0	1
Unsatisfactory	10	0	0	5	2	2	1

adjacent normal tissue, whereas the shape of tumor spectra did not markedly differ from that of normal mucosa. Mean autofluorescence spectra of all 36 patients for both malignant and innocuous oral mucosa are depicted in Figure 3. The box-plot in Figure 4 shows mean autofluorescence intensities at 511 nm as well as the median and several percentiles (from bottom to top: 5%, 10%, 25%, 75%, 90%, 95%) for all patients examined. The Wilcoxon-test reveals a highly significant difference ($p < 0.0001$) between the two groups.

In Figure 5, the averaged autofluorescence spectra (see Figure 3) are standardized and smoothed. Minima in the autofluorescence spectra at 540 and 575 nm correspond to absorption peaks of oxygenated hemoglobin (modified from

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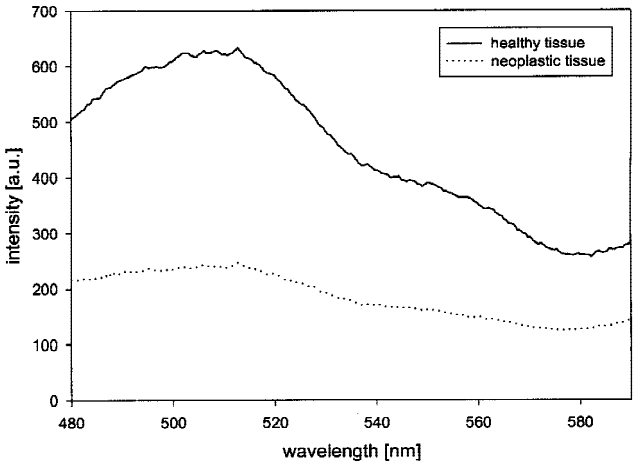

Fig. 3. Averaged autofluorescence spectra from 36 patients for malignant lesions (dotted line) and adjacent host tissue (solid line).

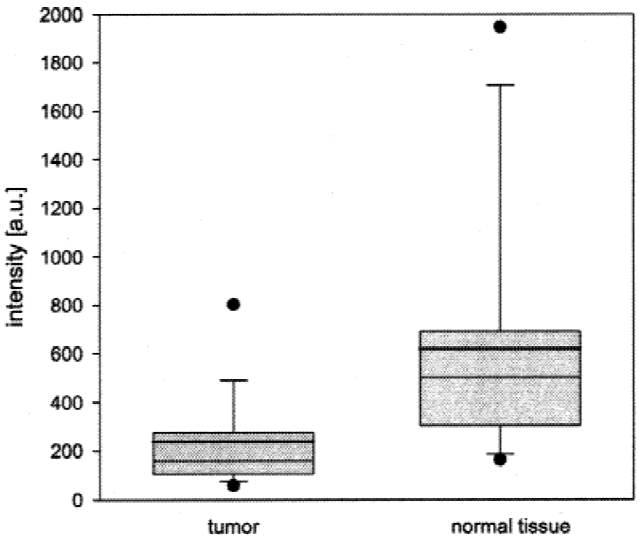

Fig. 4. Mean autofluorescence values for 36 patients (bold line) at 511 nm. The lower and upper boundaries of the boxes indicate the 25th and 75th percentile, respectively. The thin black line within the box marks the median. Error bars indicate the 10th and 90th percentiles, and black dots denote the 5th and 95th percentiles. The Wilcoxon-test showed highly significant differences ($p < 0.0001$) for both groups.

Reference 42). For non-malignant tissue the influence of blood absorption is slightly stronger than for malignant tissue.

Fluorescence ratios of host- and adjacent neoplastic tissue were calculated over a wide spectral range (470 to 600 nm) for each patient and then averaged. As a result, a maximum factor of 3.47:1 is found at 521.5 nm between normal and malignant mucosal areas (Fig. 6). This factor is almost constant for the spectral range from 520 to

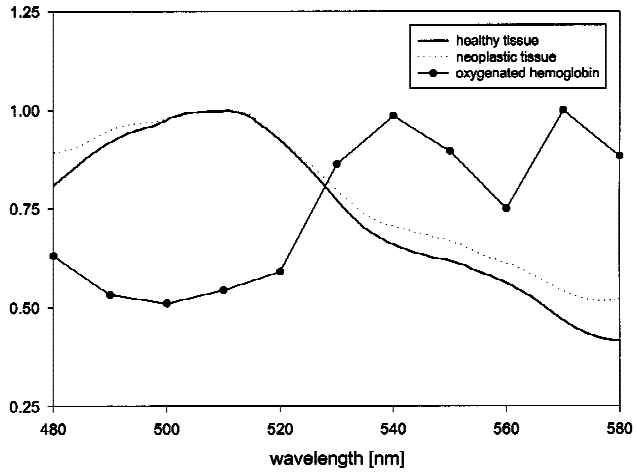

Fig. 5. Combined plotting of a standardized blood absorption spectrum of oxygenated hemoglobin (adapted from Reference 42) and the two fluorescence spectra presented in Figure 3 (standardized and smoothed). They both show inverse characteristics for the shape of their curves.

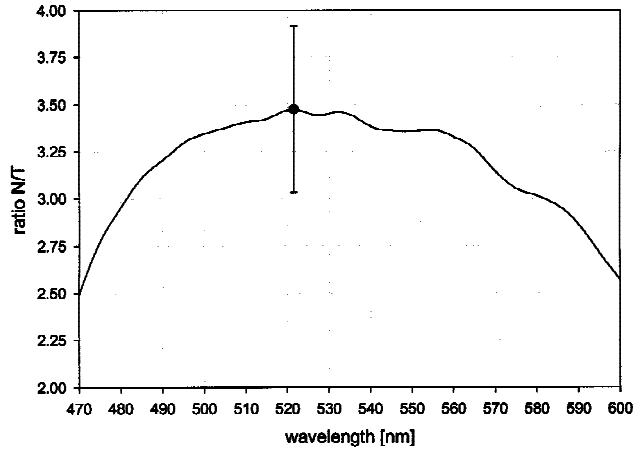

Fig. 6. Mean values of the fluorescence ratios between healthy- and adjacent neoplastic tissue for the spectral range of 470 to 600 nm. The error bar indicates the standard error.

535 nm and decreases steadily to 2.5:1 for both the blue-shifted 470 nm and the red-shifted 600 nm.

For 17 patients, spectra taken from 4 standard screening points in the oral cavity (hard palate, buccal mucosa, back of tongue, inner lip) were averaged first intra- and then interindividually; the result being depicted in Fig. 7. Highest overall autofluorescence intensities can be found at the hard palate, whereas the inner lip shows only very moderate levels of intensity. Only the spectra from the dorsums of tongue evidently differ from the other three averaged spectra.

The same data served to produce the graph

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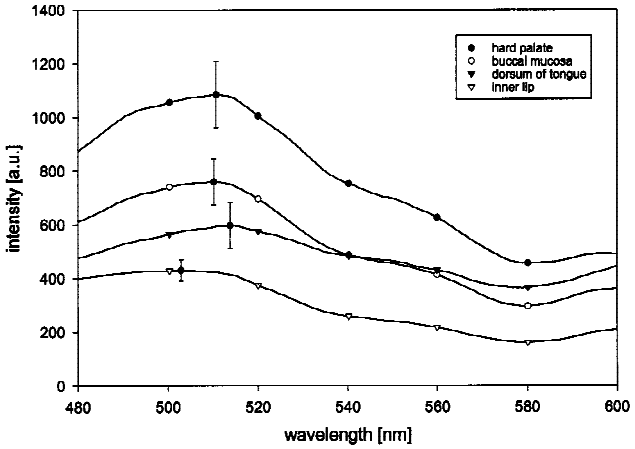

Fig. 7. Averaged autofluorescence spectra from 17 patients at 4 different locations (hard palate, buccal mucosa, dorsum of tongue, inner lip). The error bars indicate the standard errors.

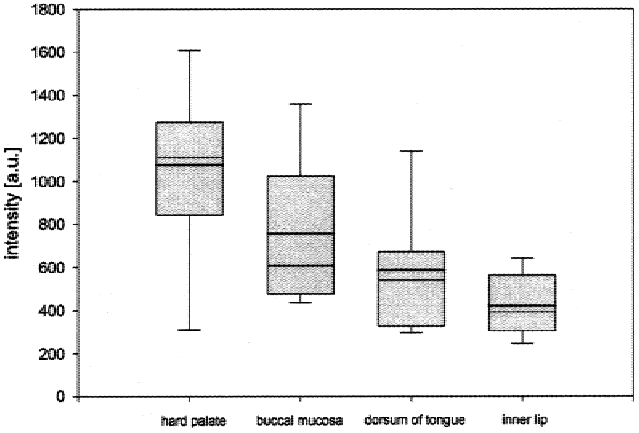

Fig. 8. Box-plot showing variations of fluorescence intensities at 511 nm at 4 oral screening points locations (hard palate, buccal mucosa, dorsum of tongue, inner lip) in 17 patients.

depicted in Figure 8, where the 4 standard screening points (mentioned above) are tested for interindividual variations in fluorescence intensities at their maximum (511 nm) for all 17 patients examined. Hereby, relatively high differences were evident at the hard palate, buccal mucosa and the dorsums of tongues, whereas the data taken from the inner lip disposed of a higher consistency.

Spectral analysis of the data generated from the healthy volunteer lead to the following result: autofluorescence intensities and characteristics vary for the different anatomical sites investigated as well as within these very areas. On the graph in Fig. 9, the fluorescence intensities at 511 nm for each area investigated are represented by box-plots. The heights of the boxes and the extents of the error bars show the degree of variations in fluorescence intensities within the same anatomical location. Factors were calculated between the 90^{th} and the 10^{th} percentile for each location—they are depicted on top of each box in Figure 9. They show a range from 1.7 for the rim of the tongue up to 4.9 for the soft palate. Highest variations in fluorescence intensities were thus evident at the soft palate—the only region of oropharyngeal mucosa investigated.

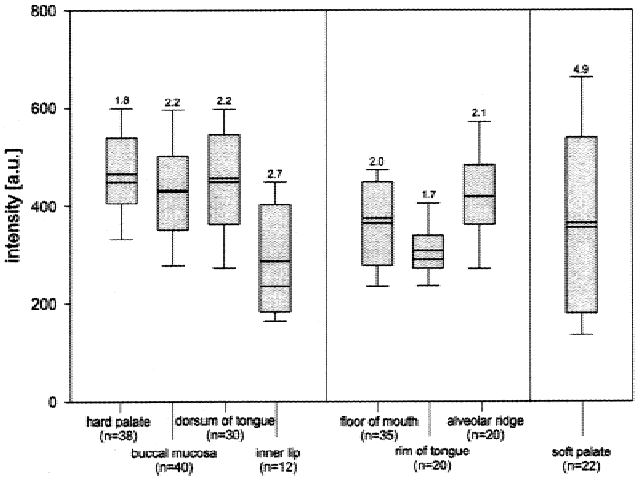

Fig. 9. Box-plot representing fluorescence intensities at 511 nm from 8 anatomical regions of the oral cavity and the oropharynx in one individual.

DISCUSSION

The prognosis for patients with oral cancer significantly rises with an early detection of the malignant lesions [4]. Even for the experienced clinician, however, currently available diagnostic methods are fairly limited, as both inspection (macroscopic and endoscopic) and conventional imaging techniques sometimes lack to provide reliable results in the detection of these flat, early tumors within the oral mucosa. Therefore, new alternative diagnostic methods in the field of oto-rhino-laryngology are sought for by numerous international research groups [7,11–14,19,20,26–28,43]. In these investigations, dyes or fluorescent markers respectively have been employed in order to achieve a better demarcation of the tumor boundaries. Still, none of these procedures has made its way into clinical routine and the medical profession generally hesitates in endorsing their use. Moreover, several other groups have endeavored to enhance the visualization of neoplastic

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changes within stratified squamous epithelium by means of autofluorescence photodetection [34–36,38–41]. Methods and results have been varying enormously and no general statements concerning the efficiency of this diagnostic procedure can be extracted.

The xenon light source we used for our investigations produces a band of excitation wavelengths from 375 to 440 nm. Its highest intensities lie within the soret band for porphyrins, and it is appropriately used for the excitation of 5-ALA induced PPIX [26–28]. However, we have noticed a subjective decrease of autofluorescence intensities in the green spectral range and irregular red fluorescing spots on the tumorous surfaces we have examined even before 5-ALA was applied. As we believe, high subjective contrasts of cancerous tissue (red) to healthy mucosa (green) after 5-ALA application [26–28] may be due to both a higher accumulation of PPIX and a decrease of green autofluorescence within the tumor. Based on these observations, we have endeavored to objectify our findings.

The only endogenous fluorophores eligible for being considerably excited at the band of excitation wavelengths we have utilized are oxidized flavins (FAD), porphyrins, elastin and partly collagen [44]. When being excited at the used band of wavelengths (λ = 375 to 440 nm), all of the fluorophores mentioned above show fluorescence emission in the green spectral range but the porphyrins, which fluoresce in a bright red color. As flavoproteins show almost no fluorescence in their reduced form, they can serve as a sensitive indicator for disturbances within the intracellular redox-system [45]. A reduction of fluorescence at the corresponding wavelengths may be a result of lower concentrations of those oxidized flavins in tumors, as proposed by Pollack and Hung [46,47]. Furthermore, elastin and collagen are mainly localized in connective tissue rather than in the mucosa. Therefore, a thickening of the mucosa in premalignant or malignant epithelial lesions might lead to an attenuation of fluorescence that emanates from those structural proteins in the submucosa (Fig. 10) [48,49]. As both the tumor centers and the lesions' borders exhibited comparable autofluorescence reductions in the green spectral range in comparison to regular oral squamous epithelium, we believe the responsible histochemical or morphological changes in tissue properties to occur early in malignant transformation. Endogenous porphyrins, on the other hand, have been controversially discussed in literature concerning their tumor-localizing properties [29–36]. A most common theory is that the observed red fluorescence is a product of microbial porphyrin synthesis, and therefore its distribution is limited to the necrotic surface of exulcerated tumors [30,33]. Our own examinations have shown a bright red fluorescence due to endogenous porphyrins on the dorsums of tongues, on gingival plaques and on the bacterial coating of necrotic parts of some of the oral neoplasms we have investigated in our previous studies [26–28]. In this present investigation, we found only one third of the tumors examined (33.3%) to be mostly covered by strongly red fluorescing material. Yet, tumor discriminating abilities were fairly limited, as the observed "red spots" that represented bacterial porphyrin accumulations did not seem to be spread homogeneously over the lesion's surfaces. Thus, we do not advocate this method for the detection of oral cancer.

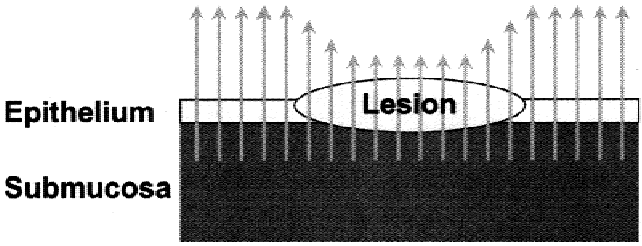

Fig. 10. Illustration showing the attenuation of fluorescence emission originating from the submucosa caused by a thickening of the epithelial layer.

Other endogenous substances affect the emission spectra by a wavelength-dependent absorption of light. It becomes apparent in Figure 5 that oxygenated hemoglobin (data taken from Roggan et al. [42]) greatly influences the shape of the autofluorescence spectra we have obtained. Of the 4 standard screening points we investigated in 17 patients (Fig. 7), blood absorption plays a similar role in all spectra but the one representing the dorsums of tongues. This may indicate that the blood supply close to the surface is lower in the dorsum of tongues than in hard palates, inner lips and buccal mucosa.

The imaging technique we used has revealed that only 43.3% of the 36 tumors investigated were subjectively well delineated from healthy oral mucosa by this autofluorescence scanning method. Yet, we found that certain locations (floor of the mouth, hard palate) are more favorable for an accurate autofluorescence tumor detection than others (rim of tongue, oropharynx), as presented in Table 1. Moreover, flat epithelial lesions

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were found to be subjectively better outlined by autofluorescence photodetection than large, exophytic tumors.

As for the picture quality, the fluorescence signals picked up by the target integrating CCD-camera were just high enough to produce clear, unblurred pictures. The use of narrow bandwidth filters, which may improve the diagnostic accuracy of the image analysis, would result in high integration times for the camera (>1/16th of a second) and a lower overall picture quality.

In spectrophotometric evaluations, even 94.4% of the tumors showed lower autofluorescence intensities than the surrounding host tissue. The averaged autofluorescence spectrum of 36 spectrophotometrically investigated neoplastic lesions was markedly lower in intensities than the one taken from healthy lining epithelium (Fig. 3). The graphs show no specific alterations in their shapes representing the spectra of normal and innocuous oral mucosa, i.e. only the intensities in the green spectral range seem to be specific for malignant and normal tissue when using violet excitation light. In Figure 4, two groups representing malignant and normal oral mucosa are formed and presented for their variations of intensities at their maximum of autofluorescence (511 nm) for all 36 patients investigated. Using the Wilcoxon-test, we were able to prove a highly significant difference ($p < 0.0001$) between the two groups. A maximum factor of 3.5:1 was calculated for the averaged fluorescence-ratios of normal to malignant epithelium (N/T ratios) at 520 nm (Fig. 6).

However, also healthy oral mucosa shows great differences in autofluorescence intensities for different locations and even within the same defined regions of the oral cavity both inter- and intraindividually. It becomes apparent in Figure 7 and 8 that the hard palate exhibits much more autofluorescence than the other 3 locations investigated. This may be due to the fact that the structural properties of connective tissue significantly differ for lining- and masticatory mucosae [50]. Whereas the lamina propria of lining mucosa (lips, cheeks, . . .) is referred to as being loose, collagen-poor connective tissue, masticatory mucosa (hard palate) boasts thick, dense bundles of collagen fibers. In Figure 8 we show that there exist high interindividual variations in fluorescence intensities for all 4 standard screening points investigated. In order to get an insight into intraindividual differences in fluorescence intensities of oral mucosa when excited with violet light, a spectral screening with a high spacial resolution (217 points of data for 8 different anatomical locations) was performed on the oral cavity of one healthy volunteer. As a result, we found great variations of autofluorescence emission in between and even within the same anatomical locations (Fig. 9). This may be a result of a variable number of elastic fibers or a varying thickness of the different layers of tissue. Variability-factors—calculated from the 90th percentile of the box plot from one anatomical region divided by the 10th percentile—ranged between 1.7 and 2.7; only for the soft palate as part of the oropharynx we found a factor of 4.9. This might indicate that the oropharynx is less suitable for the presented diagnostic method than the oral cavity.

Using the presented method, our group was able to precisely outline almost half of the tumors investigated by camera-based autofluorescence photodetection. Spectral measurements even yielded a detection rate of nearly 95%. However, autofluorescence spectra of healthy oral mucosa also highly vary in intensities for different and even within the same anatomical region of the oral cavity, which may highly affect the value of spectral data obtained. It might suggest that a maximum ratio of nearly 3.5 for fluorescence intensities between normal and malignant oral mucosa (depicted in Fig. 6) cannot be truely satisfying for an accurate tumor detection alone—at least morphological tissue criterias should be additionally considered by the examining surgeon and should yield better results.

Nevertheless, there might be two major ways by how the objective contrast in autofluorescence intensities between malignant and healthy oral mucosa is already being or could be exploited in the future:

1. The reduction of green tissue autofluorescence in neoplastic lesions of the oral cavity seems to add to the good results we've obtained using PPIX-fluorescence, which is accumulated in neoplastic tissue after exogenous application of 5-aminolevulinic acid [26–28]. As the visual impression of green in tumorous tissue is lower than in healthy tissue, the impression of red in the tumors (which is due to PPIX-fluorescence) becomes much stronger for the naked eye. Therefore, the visual contrast between tumors (red) and normal oral mucosa (green) following application of 5-aminolevulinic acid is supposed to be

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at least partly due to the reduction of green tissue autofluorescence in carcinomas.

2. As a further perspective for the future, we propose the formation of pseudo-images from a ratio of red to green fluorescence following topical application of 5-ALA. This may lead to a further improvement of the method's specificity, as two tumor-specific "markers" are combined and the procedure becomes nearly independent of the distance and the angle the tissue is examined from. Moreover, combined fluorescence photodetection would represent an easily conductible, practical, well-tolerated and reproducible method on an outpatient basis. To assess the value of this combined diagnostic procedure, first experiments are currently being performed.

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