Detection of oral squamous cell carcinoma with ex vivo fluorescence confocal microscopy: Sensitivity and specificity compared to histopathology

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Abstract
Real-time microscopic imaging of freshly excised tissue enables a rapid bedside-pathology. A possible application of interest is the detection of oral squamous cell carcinomas (OSCCs). The aim of this study was to analyze the sensitivity and specificity of ex vivo fluorescence confocal microscopy (FCM) for OSCCs and to compare confocal images visually and qualitatively with gold standard histopathology. Two hundred eighty ex vivo FCM images were prospectively collected and evaluated immediately after excision. Every confocal image was blindly assessed for the presence or absence of malignancy by two clinicians and one pathologist. The results were compared with conventional histopathology with hematoxylin and eosin staining. OSCCs were detected with a very high sensitivity of 0.991, specificity of 0.9527, positive predictive value of 0.9322 and negative predictive value of 0.9938. The results demonstrate the potential of ex vivo FCM in fresh tissue for rapid real-time surgical pathology.

KEYWORDS
fluorescence confocal microscopy, oral cancer, oral squamous cell carcinoma, rapid pathology

1 | INTRODUCTION

Oral cancer is the sixth most commonly diagnosed cancer in the world [1]. Early detection and precise removal of oral squamous cell carcinomas (OSCCs) is essential for optimal therapy and prognosis of the patients [2].
evaluation or from fresh surgical excisions and biopsies [3–9]. Confocal microscopy is a technical tool for optical sectioning of specimens that provides images with high resolution without tissue damage, which are similar to conventional histopathology. These images have been shown to have similar features with histopathology, including high resolution, large fields of view and contrasting imaging modes [9]. The technology of ex vivo FCM combines two different lasers with wavelengths of 488 nm (fluorescence mode) and 785 nm (infrared, for reflectance mode) [10]. The FCM used in this work embodies improvements with respect to a previous generation of the device, which was much slower and generated only black-and-white images in a single fluorescence mode. A fluorescent contrast agent (acridine orange) stains nuclei and increases the contrast of nucleated cells, which are highlighted by the blue laser. Additionally, the reflectance laser is used for structural information of the sample. The built-in algorithm translates the signal into pseudocolored images similar to hematoxylin and eosin (H&E) [11]. Confocal mosaics display up to 25 × 25 mm² of tissue in less than 1 minute that correspond to a view with ×2.5 magnification in the standard light microscopy. This technology enables therefore an intraoperative real-time assessment of freshly excised tissue [2]. An example of this procedure was recently demonstrated by Rocco et al. for radical prostatectomy with an ex vivo FCM [12]. In a study by Ragazzi et al. [13], a previous generation of FCM was tested on solid tissues and organs: breast, lymph node, thyroid and colon. For the first time, this work showed high accuracy of confocal images of nonskin malignancies compared with the corresponding histological sections.

Recent studies have demonstrated an excellent correlation between state-of-the-art ex vivo FMC images and histopathology for basal cell carcinomas [14, 15]. In a study by Karen et al. [6], a sensitivity of 97% and specificity of 89% in detected basal cell carcinomas with FCM have been shown for 149 submosaics. Until now, to the best of the authors’ knowledge, there are no studies showing an ex vivo FCM application in oral cancers. Recently, an in vivo reflectance confocal microscope has been used to describe the microscopic features of oral carcinoma [16].

The primary aim of this study is to validate the diagnostic accuracy of ex vivo FCM against gold standard histopathology; to calculate prospectively the sensitivity (S), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) in detecting OSSC. A secondary aim is a qualitative evaluation of confocal images and visual comparison with corresponding histopathology.

2 MATERIALS AND METHODS

From September 2019 to January 2020, 70 oral lesions were surgically removed in a single-institutional observational cohort study with a prospective design. The study protocol was reviewed and accepted by the ethics committee for clinical studies of the Heidelberg University (registry number S-665-2019). The inclusion criteria were (a) patients aged 18 years and older who gave a written informed consent (or their parents, if the patient was younger than 18 years); (b) participants diagnosed as having a suspicious oral mucosal lesion; (c) a biopsy or resection of lesion was indicated. The exclusion criteria were as follows: (a) recurrent pathology of oral mucosa and (b) previous surgery or other treatments. The mucosal excisions were rinsed in isotonic saline solution, immersed in acridine orange solution (1 mM) for 20 seconds and rinsed again for another 5 seconds. The examination was then done using an ex vivo FCM (Vivascope 2500 Multilaser, Lucid Inc., Rochester, New York) in combined reflectance and fluorescence mode. The details of the applied confocal mosaic processing and acquisition have been described earlier [5, 17]. Acridine orange indicates the nuclei and provides a strong nuclear-to-cytoplasm and nuclear-to-dermis contrast and the use of it does not affect histopathology, as shown in a study by Gareau et al. [5]. The mosaics were displayed on a large monitor with high resolution to mimic the standard ×2.5 view of histopathology. Seventy mosaics with both positive and negative cases of OSCC were prepared for this study. Mosaics were divided into four smaller submosaics to provide morphologic characteristics at higher resolution and magnification. All images were reviewed blindly to histopathological diagnoses and to interpretations of other physicians. The physicians included two maxillofacial surgeons and a pathologist. The confocal submosaics were displayed in a random order at magnifications of ×2 to ×4 on a 23-inch flat-screen monitor (EIZO T2381W, LCD-Monitor). Submosaics were zoomed in for precise examination of images. The field of view of single images was 550 × 550 μm² and the maximum mosaic area was 25 × 25 mm².

Each mosaic was evaluated for presence or absence of OSCC based on shape, size, nuclear atypia or pleomorphism, as well as increased nuclear density. The analysis of ex vivo confocal images was further based on preliminary ex vivo and in vivo knowledge from published data and, in case of the pathologist, based on histopathological experience. Confocal features of OSCC have been previously described in an in vivo study by Maitland et al. [16].
Statistical analyses were performed with SPSS 25.0 (IBM, Armonk, New York) and R, version 3.6.2. The outcome in this study was sensitivity, specificity, PPV and NPV for the evaluation of the presence or absence of OSCC in single submosaics, and by comparison of results with histopathology. True-positive was defined as the presence of tumor in both the histopathology and confocal images; true-negative as no tumor in both; false-positive as the presence of tumor in confocal imaging but no tumor in the histopathology; and false-negative as the presence of tumor in histopathological sections which was not observed in confocal imaging. All confidence intervals were computed by the formula of Wilson [18].

## RESULTS

In this study, ex vivo images of 70 oral lesions from 70 patients were evaluated. To each FCM pattern, a corresponding H&E histopathology was done. Table 1 shows patient and tumor data. Each confocal mosaic image was halved into four submosaics and displayed with an approximate magnification of ×550. From 70 mosaics, 280 submosaics were created. One hundred eleven (39.6%) submosaics contained OSCC tumor and 169 (60.4%) were either benign or healthy tissue. An example of a sample with OSCC is shown in Figure 1. The examples of a submosaic with OSCC and singular tumor cells are shown and described in Figure 2.

The overall S, Sp, PPV and NPV in detecting OSCC in surgical samples were 91.6%, 75.4%, 83% and 87.8%.

### Table 1

| Patients |   |
|----------|---|
| Age (y)  | Average 68.7 |
| Gender (%) | |
| Female   | 47.8 |
| Male     | 52.2 |
| Lesion   | |
| OSCC     | 51 |
| Benign   | 49 |
| Location (%) | |
| Lip      | 7 |
| Palate   | 18 |
| Tongue   | 37 |
| Buccal mucosae | 15 |
| Floor of mouth | 23 |

**Abbreviation:** OSCC, oral squamous cell carcinoma.

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**FIGURE 1** Example of an oral squamous cell carcinoma mosaic in ex vivo fluorescence confocal microscopy

**FIGURE 2** A-C, Examples of oral squamous cell carcinoma submosaics and D-H, singular tumor cells in ex vivo fluorescence confocal microscopy: A, disturbed polarity of the basal cells, B, basal cell hyperplasia, C, irregular epithelial stratification or disturbed maturational sequence, D, cellular pleomorphism/anisocytosis, E, nuclear hyperchromatism, F, prominent nucleoli, G, intraepithelial keratinization, H, increase in nuclear cytoplasmic ratio
The average sensitivity and specificity of detecting OSCC were different among the two physicians (expert and novice) and the pathologist. The highest diagnostic accuracy was identified in evaluation through the pathologist, followed by physician 2 (maxillofacial surgeon trained in histopathology) and physician 1 (maxillofacial surgeon trained in in vivo RCM).

Results of the statistical analysis and evaluation through the three reviewers are given in Tables 2-5.

### TABLE 2
**Statistical evaluation: Blind comparison of FCM mosaics to the corresponding H&E histopathology**

| Parameter                        | Pathologist            | Physician 2 (expert) | Physician 1 (novice) |
|----------------------------------|------------------------|----------------------|----------------------|
| Sensitivity (95% CI)             | 0.991 (0.951, 0.998)   | 0.964 (0.911, 0.986) | 0.982 (0.937, 0.995) |
| Specificity (95% CI)             | 0.9527 (0.909, 0.976)  | 0.6627 (0.589, 0.73) | 0.568 (0.493, 0.64)  |
| Positive predictive value (95% CI)| 0.9322 (0.872, 0.965) | 0.6524 (0.577, 0.721) | 0.5989 (0.526, 0.667) |
| Negative predictive value (95% CI)| 0.9938 (0.966, 0.999) | 0.9655 (0.915, 0.987) | 0.9796 (0.929, 0.994) |

Note: The numbers in parentheses correspond to the 95% confidence intervals (CI). Cohen’s kappa statistic [19]: pathologist vs specialist 1: 0.554849, pathologist vs specialist 2: 0.5225102, specialist 1 vs specialist 2: 0.8343373.

Abbreviation: FCM, fluorescence confocal microscopy.

### TABLE 3
**Detection of OSCC in samples with ex vivo FCM (pathologist)**

|               | H&E + | H&E − | N (%) |
|---------------|-------|-------|-------|
| Ex vivo FCM + | 110   | 8     | 118 (42%) |
| Ex vivo FCM − | 1     | 161   | 162 (58%) |
| N (%)         | 111 (39.6%) | 169 (60.4%) | 280 (100%) |

Abbreviations: FCM, fluorescence confocal microscopy; OSCC, oral squamous cell carcinoma.

### TABLE 4
**Detection of OSCC in samples with ex vivo FCM (physician 1, expert)**

|               | H&E + | H&E − | N (%) |
|---------------|-------|-------|-------|
| Ex vivo FCM + | 107   | 57    | 164 (58.6) |
| Ex vivo FCM − | 4     | 112   | 116 (41.4) |
| N (%)         | 111 (39.6%) | 169 (60.4%) | 280 (100%) |

Abbreviations: FCM, fluorescence confocal microscopy; OSCC, oral squamous cell carcinoma.

### TABLE 5
**Detection of OSCC in samples with ex vivo FCM (physician 2, novice)**

|               | H&E + | H&E − | N (%) |
|---------------|-------|-------|-------|
| Ex vivo FCM + | 109   | 73    | 182 (65%) |
| Ex vivo FCM − | 2     | 96    | 98 (35%) |
| N (%)         | 111 (39.6%) | 169 (60.4%) | 280 (100%) |

Abbreviations: FCM, fluorescence confocal microscopy; OSCC, oral squamous cell carcinoma.

4 | DISCUSSION

OSCC at early or preneoplastic stages may be difficult to recognize based on clinical examination alone. The standard technique in OSCC surgical treatment is a wide resection with at least 1 cm safe margins in all directions from the visible tumor. In case of advanced tumor, difficulties in differentiation of cancerous and normal tissue may lead either to extensive defects, or incomplete resection with reoccurrence potential. In case of non-insane resection, as diagnosed through intraoperative frozen pathology, there are often difficulties in finding the corresponding locus in the wound.

Although surgical excision and histopathology still represent the diagnostic gold standard in oral oncology, alternative methods for rapid tissue assessment have to be evaluated. To our knowledge, this is the first study to establish, objectively and quantitatively, the feasibility of confocal laser scanning microscopy for detection of OSCC in excised specimen.

S and Sp were as high as 0.991 and 0.9527, respectively, for detection of OSCC by a pathologist. Based on histopathological findings of OSCC features, the examination of confocal mosaics was similar to the routine histopathology. A rapid observation of tissue architecture at low resolution, followed by higher magnification when needed was performed through a pathologist and two physicians. Overall, the correlation between the confocal pictures and histopathology was good. From 111 true-positive confocal images of OSCC proven by histopathology ex vivo picked up 110, 109 and 107 OSCCs (pathologist, physicians 1, and 2). When reviewing false-negative cases, most of them represented well differentiated OSCCs histopathologically.

On the other hand, from 169 negative mosaics, 8, 57 and 73 were false-positive in the ex vivo FCM
investigation. These data may be explained by the fact that patterns of noninvasive dysplastic tissue, leukoplakia or lichen might mimic high-grade dysplasia and be therefore interpreted as an OSCC. Another reason is a particularity of ex vivo FCM scanning where a tissue sample is often not orientated as in a histopathological cut and scanned either from its upper/mucosal side or from the lower/dermal layer. A classic histopathological vertical cut through multiple levels was not always performed here, as the primary question of investigation was the possibility of quick bedside investigation without specific tissue preparation.

In the present study, a clear correlation of interpretation accuracy and the expertise level was shown. Both the physicians have no histopathological background. As expected, best results of mosaic interpretation were presented by the pathologist followed by physician 2, who was experienced in histopathology of OSCCs, and physician 1, who was trained in in vivo RCM only.

A number of previous works show the application of ex vivo FCM for diagnosis of neoplasia. In an investigation by Longo et al. [10], an application of ex vivo FCM for diagnosis of cutaneous squamous cell carcinoma has been shown. In 2016, the same working group [20] presented an application of ex vivo confocal microscopy for preliminary diagnosis of suspicious conjunctival lesions. Although in both works, the confocal images showed good histological details and allowed margin assessment; the technology was limited by some qualities of a previous version of ex vivo FCM. These are: slower acquisition, smaller field of view, black-and-white images of fluorescence mode only. The new version achieves higher performances through increasing 3-fold the field of acquisition and the velocity with which it is acquired, as well as improving digital performance through transforming previous black-and-white images into colored images, which mimic H&E staining [21].

In the work by Puliatti et al., the ex vivo FCM was applied for real-time examination of prostatic tissue [21]. In the evaluation of specimens through three pathologists, the overall diagnostic agreement between ex vivo FCM and histopathological diagnoses was substantial with a correct diagnosis in 91% of the cases (κ = 0.75) and an area under the curve of 0.884 (95% confidence interval 0.840-0.920), 83.33% sensitivity and 93.53% specificity. The results are similar to those presented in our work.

A limitation of the present study is the absence of established criteria for detecting OSCCs in ex vivo FCM. Up to date, only the diagnostic criteria specifically elaborated for H&E stained tissue samples as well as preliminary data of in vivo RCM OSCC imaging are available and were used on the mosaic evaluation. These histological criteria are: drop-shaped rete pegs, disturbed polarity of the basal cells, cellular pleomorphism and anisocytosis, basal cell hyperplasia, irregular epithelial stratification, disturbed maturational sequence and intraepithelial keratinization, nuclear hyperchromatism, increase in nuclear cytoplasmic ratio, and loss of cellular adhesion and cohesion.

Maitland et al. [16] could show disarranged nuclei, architectural disarray, irregular polarization of the cell layers, keratinocytes with different boundaries and cytoplasmic refractivity at the upper and lower layers of OSCC tissue using an in vivo RCM prototype.

There is no evidence that these criteria would be specific or optimal for the diagnosis and detection of OSCC in ex vivo confocal mosaics.

In conclusion, the potential of ex vivo FCM in oral oncology may be an attractive completion to standard histopathology and represents a first step toward a real-time peri- and intraoperative examination, and further automated tissue classification or 3D-pathology. The potential benefit of rapid intraoperative pathology during resection of OSCC is a more precise definition of margins and areas of non-in-sano resection. This may result on the one hand in fewer recurrences, and on the other hand in less postoperative morbidity and better functional outcome (speech and swallow) and reduced costs for extemporary pathology.

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CONFLICT OF INTEREST
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