Ex vivo fluorescent confocal microscopy images of oral mucosa: Tissue atlas and evaluation of the learning curve

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Funding information
Bundesministerium für Bildung und Forschung, Grant/Award Number: 13GW0362D

Abstract
Ex vivo fluorescence confocal microscopy (FCM) is a developing tool providing rapid digital imaging of fresh tissue utilizing high-resolution optical sectioning that highly corresponds with conventional haematoxylin and eosin (H&E)-stained slides. A very little data on oral mucosa lesions exist currently. The present work aimed to create an image atlas of benign and malignant oral tissues and compare them to the corresponding histopathology. Furthermore, we aimed to evaluate the learning curve for confocal image interpretation. From 50 samples obtained from the oral mucosa, including oral squamous cell carcinoma (OSCC), dysplasia, and healthy oral tissue, ex vivo FCM images and corresponding H&E slides were created and collected into a tissue atlas. Additionally, two experts were asked to analyze the images to assess the learning curve. Ex vivo FCM images revealed high comparability with histopathological images. Tissues including OSCC, dysplasia, and healthy oral tissue, ex vivo FCM images and corresponding H&E slides were created and collected into a tissue atlas. Additionally, two experts were asked to analyze the images to assess the learning curve. Ex vivo FCM images revealed high comparability with histopathological images. Tissues including OSCC, dysplasia, and normal oral mucosa were implemented in the image atlas to provide the diagnostic fundamen for pathologists and surgeons; the learning curve was short. Future studies on this topic will be advantageous for the development of artificial intelligence-based diagnostic approaches. The current work provides a novel set of data that are structured as an atlas of common pathologies of the mucosa to enhance the existing knowledge and material on confocal images.

KEYWORDS
ex vivo fluorescent confocal microscopy, confocal, diagnosis, diagnostic, digital pathology, OSCC, oral cancer

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1 | INTRODUCTION

Ex vivo fluorescence confocal microscopy (FCM) is a real-time imaging technique allowing a high-resolution view of fresh, nonfixed tissues. It is based on a combination of two laser light sources: one with the reflectance mode (785 nm) and the other using fluorescence mode (488 nm). The first reflects the differences of the refractive indices of cellular structures, and the second allows the visualization of cellular structures through enhancement of signal with a contrast agent [1].

The fluorescent laser signal is being transformed into a blue color through digital staining. In contrast, the reflectance mode laser signal is transformed into a purple color, leading to hematoxylin and eosin (H&E) stained like image presentation when both laser signals are combined [2, 3].

The application of ex vivo FCM has been described on various tissues: in healthy skin [4], basal cell carcinoma [5], squamous cell carcinoma [6], melanoma [7, 8], and inflammatory skin lesions [9]. Further, surgical specimens of breast, lymph node, thyroid, and colon neoplastic tissues were well distinguishable in ex vivo FCM images [10]. Several investigations aimed to introduce technologies for immediate intraoperative or bed-side pathological examination of oral mucosa tissue, including frozen section, light reflectance spectroscopy, multiphoton microscopy, confocal laser endomicroscopy, and others. In our previous works [11, 12], we described the features of oral squamous cell carcinoma (OSCC) in ex vivo FCM. Although histopathology is considered the gold standard for diagnosing benign and malignant lesions of the oral mucosa, there is an increased interest in real-time, bed- or chair-side, or even intraoperative pathological tissue examination during biopsies or cancer surgeries.

The present study aimed to expand the existing knowledge on ex vivo confocal imaging by creating an image atlas of normal oral mucosa tissue and the most common pathologies, including OSCC and dysplasia.

Furthermore, we aimed to demonstrate different architectural structures in oral mucosa confocal images, such as muscles, vessels, adipose tissue, inflammatory changes, and signs of malignant transformation.

Finally, we sought to assess the learning curve of this technology to evaluate the feasibility of implementing ex vivo FCM image diagnosis in daily practice.

2 | MATERIALS AND METHODS

This study was approved by the Ethical Committee (S-665-2019), and written informed consent was obtained from all patients. The study was conducted from September 2020 to April 2021 at the Heidelberg University Medical Center, Germany. Inclusion criteria were adult patients (>18 years) who gave a written informed consent; patients with an indication for incisional biopsy of an oral lesion. Exclusion criteria were previous treatments of lesions and recurrent lesions of the oral cavity. Clinical data of included patients were collected and pseudonymized.

A total of 50 mucosal specimens were collected during oral cancer surgery procedures or the routine ambulant biopsy. Immediately after resection, the samples were brought to the Institute of pathology. Representative areas, including areas with OSCC, clinically normal tissue, and regions with an unclear presentation, were prepared by an experienced specialist of the Pathological Department and prepared for an ex vivo FCM scanning procedure. First, we stained the samples with acridine orange (0.6 mM (Sigma-Aldrich®) according to the standard protocol: 10 to 30 seconds aceto-whitening in 10%-citric acid (Sigma-Aldrich), rinsing in phosphate-buffered saline (Sigma-Aldrich) for 30 seconds, followed by incubation in acridine orange for another 30 seconds, and finally washing in physiological saline solution. We used a device/method for tissue flattening to achieve an optimal optical visualization and minimize variability in the sample's density and constitution. The technique was described in detail previously [13]. All air bubbles from the tissue samples were removed by placing them on the slide and covering them with a sponge. The cover was placed on the slide in the next step and glued magnets fixed both together. We used thinner sponges and magnets for our samples, as the samples were relatively small/not thick. The VivaScope® 2500 (Mavig GmbH, Munich, Germany; Caliber I.D.; Rochester NY, USA) was utilized for image acquisition. The device combines two laser modes: reflectance (with 785 nm wavelength) and fluorescence (with 488 nm wavelength). The combination of both was required for imaging acquisition. As described previously, the grayscale fluorescence and reflectance mosaics were digitally stained in colors according to H&E-stained images: purple color linked to cellular nuclei and pink—for noncellular structures. The device allows a maximum scanning area of 25 × 25 mm with a magnification of up to 550 and a maximum examination depth of 200 μm and vertical resolution of up to 4 μm. The average time of staining and imaging was under 3 to 4 minutes. The software used in the study was a VivaScan® (Version 11.0.1140 Mavig GmbH; Caliber I.D) and VivaBlock®.

After ex vivo FCM examination, all representative samples were immersed in formalin for fixation and further histopathological examinations. All confocal images were stored and then randomly presented to two independent specialists to assess the learning curve. After a 30 days interval, both specialists were asked to re-evaluate images. The image evaluations included an analysis of cancerous tissue within tissue structures (vessels, glands, follicles, fat, and muscle tissue), as well as benign inflammatory or dysplastic changes.
Pathological slides were compared with corresponding ex vivo FCM, and concordant and discordant tissue regions were analyzed.

### 2.1 Statistical analysis

Cohen’s kappa was applied to estimate agreement between histopathological and ex vivo FCM diagnosis [13]. Levels of agreement were defined as follows: no agreement ($\kappa < 0$), slight agreement ($\kappa = 0.01-0.20$), fair agreement ($\kappa = 0.21-0.40$), moderate agreement ($\kappa = 0.41-0.60$), substantial agreement ($\kappa = 0.61-0.80$), and almost perfect agreement ($\kappa = 0.81-0.99$). Sensitivity, specificity, and the area under the receiver operating characteristic curve (ROC) were calculated for diagnostic evaluations. Calculations were performed using Stata Statistical Software Release 15 (StataCorp. 2011, College Station, TX, USA).

### 3 RESULTS

While the paraffin-embedded, sliced, and HE-stained tissue samples showed clear borders without relevant artifacts, the obtained confocal images of the freshly excised and still damp tissue samples revealed artifacts that are due to both tissue fluid and the applied staining solution. Consecutively artifacts like air bubbles and freely floating cell material were also seen (Figure 1).

All resected tissue biopsies were stratified into three main groups: OSCC, dysplasia, or normal tissue (Figures 2-4). H&E-stained tissue images are shown on the left side. The corresponding images obtained via FCM scanning are displayed on the right.

#### 3.1 Oral squamous cell carcinoma

In conventional histopathology, OSCC is characterized by tumor cells destroying the basal membrane layer and invading the subcutaneous tissue. The tumor cells can now appear as singular cells or in the form of tumor islands. Depending on their grade of differentiation, tumor cells can look more or less similar to physiologic epithelial cells and can produce keratin pearls or show cytological atypia, nuclear and cellular pleomorphism, hyperchromasia, and elevated levels of mitotic figures as a sign of increased proliferation.

These features could also be observed in the obtained ex vivo FCM images, as described in previous studies of our workgroup [11, 12].

The following section includes representative examples of the obtained ex vivo FCM images with their corresponding H&E-stained pendants. The compared OSCC characteristics involved disturbed polarity of the basal cells (Figure 4), ulcerations and invasion of the underlying tissue in the form of noncohesive tumor cell islands (Figures 2 and 4), typical tumor islands (Figures 2-7) and accompanying inflammatory reactions and cellular pleomorphism (Figures 2-5), as well as nuclear hyperchromatism, prominent nucleoli and an increase in the nuclear-cytoplasmic ratio (Figures 5-7).

#### 3.2 Dysplasia

The ex vivo FCM features of dysplasia typically included both architectural and cytological changes. Architectural signs of dysplasia were asymmetrical epithelial stratification, an increased number of mitotic figures in the epithelium, dyskeratosis, drop-shaped rete pegs, and keratin pearls within these rete pegs, loss of polarity of basal cells, and basal cell hyperplasia or anaplasia. The cytological features of dysplasia were...
nuclear pleomorphism, cellular pleomorphism, increase in the nuclear-cytoplasmic ratio, prominent nucleoli, and hyperchromasia (Figure 5, epithelial layer close to the ulcerous lesion).

3.3 | Normal tissue

Different entities of normal tissue were also examined both in H&E-stained scans and in ex vivo FCM obtained
images. As expected, here as well, a high correspondence could be observed. A comparison of physiological epithelial layers can be seen in Figures 8, 9 and 12. In contrast to paraffin-embedded, H&E-stained tissues, the keratin layer eludes in some FCM images, which could be a result of pressureless positioning of the fresh tissue samples.

Figure 10 shows corresponding images of seromucous glands with typical ductal and lobular features. The so-called Ebner-Demilunesformations that can typically be seen in
H&E-stained seromucous glands were not as evident. This may be due to the fact that the freshly excised tissue does not undergo any squeezing and therefore does not show these artifacts when placed in the ex vivo FCM scanner.

Figure 11 shows representative samples of muscle fibers of the tongue. Typical features of H&E-stained, skeletal, muscular fibers can be seen in these transverse and longitudinal cross sections (Figures 12 and 13).
3.4 Learning curve assessment

Both specialists achieved satisfying levels of agreement in evaluations of confocal and histopathological images of the oral mucosa (Table 1). From 50 collected samples, 35 contained OSCC, and 15 specimens, including the healthy tissue and dysplastic mucosa, were considered as a control (H&E negative group). As expected, the agreement increased, reaching 97% in the second evaluation (compared to 89% in the first round for both experts). The reproducibility was excellent the second time. The specificity/sensitivity for correct diagnosis in FCM images achieved 94.3%/86.7% and 97.1%/93.3% for the first evaluation, and 91.4%/80.0% and 100%/93.3% for the second (first and second rater, respectively).

4 DISCUSSION

The present study provided a series of confocal images with corresponding histopathological slides of oral mucosa specimens. These included normal, dysplastic, and cancerous tissue, freshly prepared and not formalin-fixed. Furthermore, the typical cellular morphology and structures were well recognizable in confocal images so that differentiation between benign and cancerous tissues was possible. The learning curve and the agreement for evaluation between the experts showed excellent results.

The ex vivo FCM of mucosal tissue has the potential to optimize the clinical and surgical routine in oral- and maxillofacial surgery. Furthermore, it might replace the frozen section analysis and represents the basis for implementing artificial intelligence-based approaches.
4.1 The application of ex vivo FCM

All steps for preparation of frozen tissue blocks, cutting, and staining are resource and time-consuming. The real-time pathological examination is investigator dependent and requires good skills. The FCM can save time, which can be very important in everyday clinical practice. In addition, the number of potential sources of error is minimized due to fewer professionals involved. With the ex vivo FCM technology, minimal tissue preparation, and basic skills for scanning are required.

In our previous works, we assessed the accuracy of this novel technology and reported a sensitivity of 0.991, specificity of 0.9527 for the detection of OSCC [11]. Moreover, benign and inflammatory changes were well recognizable. The alternative techniques applied in the oral region are light reflectance spectroscopy [14], multiphoton microscopy [15], confocal laser endomicroscopy [16].

To integrate a new technology successfully in clinical routine, the learning curve of image interpretation needs to be investigated [17, 18]. In our study, two experts were involved in the learning curve evaluation. The similarity of confocal scans with histopathology is supported by the good agreements between first and second image evaluations.

Concerning the learning curve and the reproducibility, classification accuracy in the first evaluation was almost as good as in the second evaluation, indicating the minor level of experience required and the robustness of the classification accuracy (low intraobserver variability).

The main limitation of the present study is a restricted number of histological entities described (no data on lichen, herpes, lupus, different bacterial or viral alterations). Furthermore, in the most confocal scans, the grading of OSCC was hardly recognizable. However, we were able to demonstrate clinically applicable diagnostics for the common pathologies. For more specific entities, larger numbers of cases are necessary to ensure a reliable validation. Considering the faster diagnosis and good diagnostic results for the included pathologies, this procedure can be extended to rarer entities in future clinical studies.

5 CONCLUSION

Ex vivo FCM is a novel digital technology for the investigation of normal and pathological oral conditions. We prepared an image atlas that expands the existing data on FCM images of OSCC, dysplasia, and normal oral mucosa and improves future image interpretation. The current study is one of the first to apply FMC in mucosal tissue and analyze as well as interpret it in terms of architectural structures [19, 20]. Ex vivo FCM implementation for diagnosis of oral mucosa pathologies is associated with a satisfying learning curve and level of agreement between examiners and is well comparable with the established gold standard histopathology. Future studies are warranted to develop the field of digital pathology and artificial intelligence-based approaches.
ACKNOWLEDGMENT
Open Access funding enabled and organized by Projekt DEAL.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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How to cite this article: V. Shavlokhova, C. Flechtenmacher, S. Sandhu, M. Vollmer, A. Vollmer, B. Saravi, M. Engel, O. Ristow, J. Hoffmann, C. Freudlsperger, J. Biophotonics 2022, 15(2), e202100225. https://doi.org/10.1002/jbio.202100225