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ABSTRACT

Usually, tissue images at cellular level need biopsies to be done. Considering this, diagnostic devices, such as microendoscopes, have been developed with the purpose of do not be invasive. This study goal is the development of a dual-channel microendoscope, using two fluorescent labels: proflavine and protoporphyrin IX (PpIX), both approved by Food and Drug Administration. This system, with the potential to perform a microscopic diagnosis and to monitor a photodynamic therapy (PDT) session, uses a halogen lamp and an image fiber bundle to perform subcellular image. Proflavine fluorescence indicates the nuclei of the cell, which is the reference for PpIX localization on image tissue. Preliminary results indicate the efficacy of this optical technique to detect abnormal tissues and to improve the PDT dosimetry. This was the first time, up to our knowledge, that PpIX fluorescence was microscopically observed in vivo, in real time, combined to other fluorescent marker (Proflavine), which allowed to simultaneously observe the spatial localization of the PpIX in the mucosal tissue. We believe this system is very promising tool to monitor PDT in mucosa as it happens. Further experiments have to be performed in order to validate the system for PDT monitoring.

Keywords: Microendoscope, proflavine, protoporphyrin IX, photodynamic therapy.

1. INTRODUCTION

Optical techniques are emerging to assist or overcome the necessity of tissue biopsy in clinical diagnosis.[1] They are mainly based on reflectance, scattering or fluorescence and can be classified on wide-field or microscopic techniques. Compared to traditional methods, optical techniques have the advantage to be faster and minimal or noninvasive.[2] Several optical measurements can be done *in situ* contributing to tissue preservation. Microendoscopy is one of these alternative or auxiliary techniques to the gold standard of biopsy and histology.

Microendoscope is a microscope system coupled to an optical fiber or fiber bundle capable of making images, with micrometer scale, *in situ.* In this equipment category, there are some different configurations including confocal and fiber-bundle microendoscope.[3] The former provides images with higher resolution using lasers[4], and the latter operates with fiber bundle in contact with the sample, resulting in wide-field images without the need of scanning.[5]

The equipment presented in this study is a fiber-bundle microendoscope based on fluorescence detection. This fluorescence could be endogenous, when it is provided by the tissue itself, or exogenous, when a constrast agent is added to the tissue to make it visible by the equipment.

Among the fluorescent labels responsible for exogenous fluorescence, proflavine is commonly used to prepare tissue for fluorescence images with subcellular-resolution due to its property of rapidly staining cell nuclei and cytoplasmic structures.[2] A microendoscope to image proflavine fluorescence was proposed by Muldoon *et al*.[6] In this present manuscript, we propose to image proflavine together with Protoporphyrin IX (PpIX) to make possible tissue characterization on microendoscope images. PpIX, naturally produced by cells, is an endogenous fluorescent molecule used to evidence disturbed cells and also as photosensitizer for photodynamic therapy (PDT).[7] PpIX production can be locally induced by using a precursor; 5-aminolevulinic acid and methyl aminolevulinate are the most used precursors. Tissues with metabolic alterations tend to have modifications in the amount of PpIX.[8] Thereby, the analysis of PpIX fluorescence can help in the identification of abnormal tissues. Other types of labels are composed of nanoparticles or based on specifics antibodies, to attach to proteins of interest, for example. To the best of our knowledge, this is the first two-channel microendoscope using Food and Drug Administration (FDA) approved labels.[9,10]

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Photodynamic therapy is a photo-chemical treatment based on the action of a specific excitation light, a photosensitizer and molecular oxygen. The light excites the drug (photosensitizer) which transfer energy to molecular oxygen producing reactive species, causing cell death. It can be used for the treatment of several types of cancer including non-melanoma skin cancer, and cervical cancer.[11,12] The treatment monitoring is possible to be assessed by fluorescence before and after each session. Before PDT, using a specific wide-field technique, PpIX fluorescence emission can be used with diagnosis purposes, since it preferentially locates in diseased cells.[7] Moreover, immediately after PDT, PpIX presence verification can evaluate its consume and, indirectly, if the therapy session was well succeeded. The later can be used to improve the treatment efficacy, through the determination of the best drug-light interval, which is an essential parameter for PDT success.

We present in this paper the assembled dual-channel microendoscope and *in situ* images obtained from volunteers stained with Proflavine and PpIX, which emits fluorescence at green and red region, respectivelly, when excited with blue light. We show the possibility to use this system as a noninvasive option to microscopically monitor PDT sessions in real-time.

2. MATERIAL AND METHODS

The dual-channel microendoscope presented here, whose set up is represented in figure 1, has an excitation light from a halogen lamp with a 460 nm short-pass filter, whose emission spectrum is shown in figure 2. This light provides excitation for both used fluorescent labels: proflavine and PpIX. An optical fiber bundle (FIGH 30-850N - Fujikura) guides the excitation light to the sample after it has been reflected by a dichroic mirror (DMLP505 - Thorlabs) and been focused by an objective lens (10x/0.25 NA - Olympus) to be coupled to this guide. The same fiber bundle collects emission light from the sample and delivers it back to the system. This light response passes through the first dichroic mirror (the same which reflects the excitation light), it is reflected by a mirror and is divided into two light beams by a second dichroic mirror (DMLP605 - Thorlabs). Each light beam passes through an optic filter (green channel: FF01-550/88 nm - BrightLine®, Semrock Inc, New York, USA and red channel: FEL0600 - Thorlabs) before being detected by a camera (FMVU 03MTM - Firefly, Point Grey) of the correspondent channel (red or green). Finally, simultaneous images from both cameras could be seen on a computer provided by a homemade algorithm on Python Software Foundation (Python Language Reference, version 2.7.14. Available at http://www.python.org).

One channel is responsible for capturing the light fromproflavine fluorescence emission (green channel), and the other channel detects the light from PpIX fluorescence emission (red channel). Proflavine is used to label mainly cell nuclei, and can be used as a spatial reference for PpIX local distribution in the tissue. The final image, given by the sum of the images of each channel, can be used to monitor PDT: distribution homogeneity of PpIX, drug-light interval and PpIX degradation after irradiation.

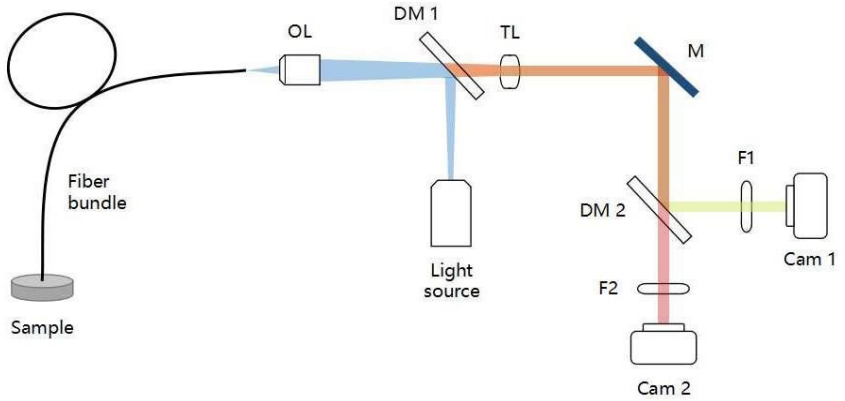

Figure 1. Dual-channel microendoscope setup. Excitation light from a halogen lamp is reflected by a dichroic mirror (DM1) and focused by an objective lens (OL) to be coupled to the fiber bundle. The emission light is collected by the same fiber bundle and is delivered back to the objective lens (OL), transmitted by the dichroic mirror (DM 1) and the tube lens (TL), reflected by a mirror (M) and divided by a second dichroic mirror (DM 2), each resultant light beam pass through a filter (F1 to green channel and F2 to red channel) before been detected by the camera (Cam1 and Cam 2).

Characterization of fluorescent labels are shown in figure 3 through the absorption and emission spectra of Proflavine hemisulfate salt hydrate (Sigma-Aldrich, USA) solution; same used to stain the image cells; and PpIX (Sigma-Aldrich, USA). The positions of absorbance maximums can be noted at same spectra range of the excitation light showed in figure 2.

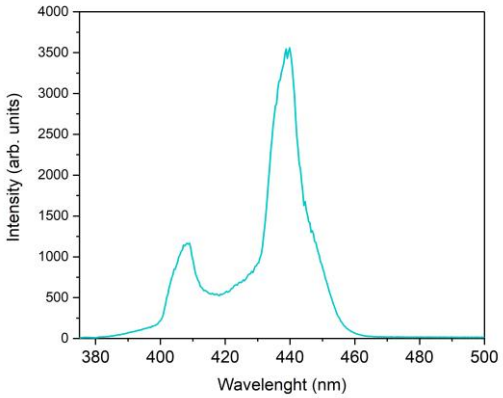

Figure 2. The emission spectrum from a halogen lamp, with a 460 nm short-pass filter, used at the microendoscope described in Figure 1.

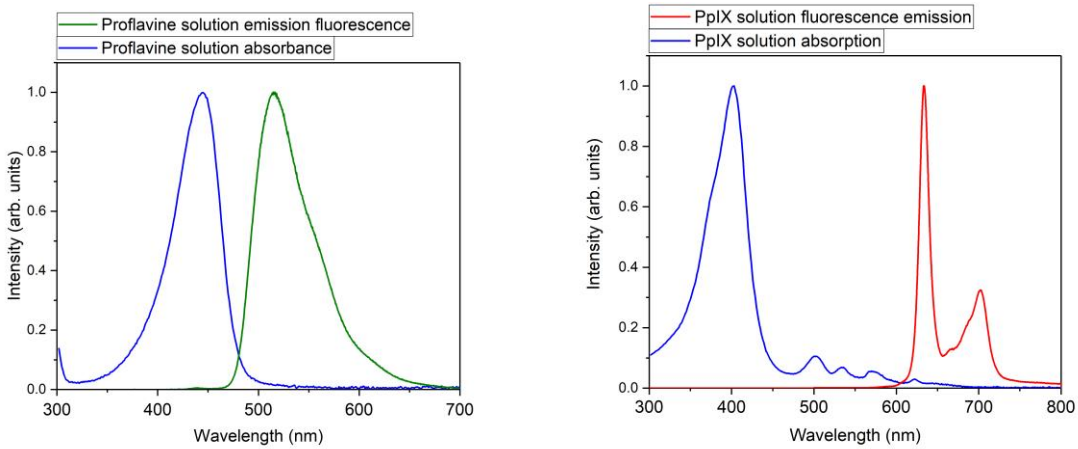

Figure 3. Absorption and emission spectra of proflavine at 200 µM in H2O (left) solution and PpIX at 90 µM in DMSO (right).

Some preliminary results were obtained imaging the healthy oral mucosa tissue of a volunteer. To increase the natural amount of PpIX on this evaluated tissue, a PpIX precursor - 5-aminolevulinic acid (ALA, PDTPharma, Brazil) - cream was used. Proflavine solution in water at a concentration of 0.005% (w/v) was applied to the oral mucosa of the volunteer to stain mainly the cell nuclei. Following 3h of ALA incubation and immediately after proflavine application on tissue, images were acquired. Form comparison, images of the same tissue were acquired with application of proflavine solution only, showing just the natural amount of PpIX.

3. RESULTS AND DISCUSSION

With the use of fiber bundle to wide-field microendoscope configuration, spatial resolution is limited by the diameter of each fiber which composes the bundle and their relative position. The fiber bundle we used has 30,000 fibers dispersed through an area with about 850 µm in diameter.

System characterization was done imaging USAF 1951 target on a fluorescent background to measure spatial resolution. Figure 4 shows the measured image of the smallest patterns on the target and the intensity profile of the sixth element of group 6 (width line of 4.38 µm), which is the smallest resolved element on this system.

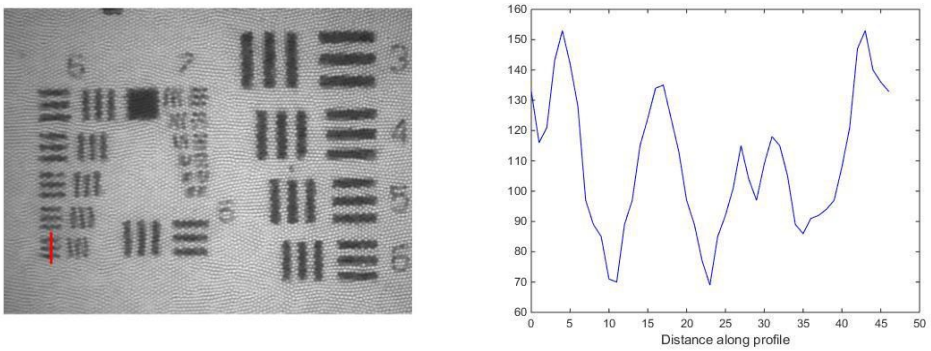

Figure 4. Image of USAF 1951 target with a fluorescent paper on background by red channel to show spatial resolution of microendoscope.

Images from each channel and their composition is presented in figure 5 and 6, of fluorescently stained tissue with proflavine solution only (figure 5) and after ALA incubation and application of proflavine solution (figure 6). The background of these images was removed to confirm the presence of the labels fluorescence, and their contrast was increased to facilitate the visualization.

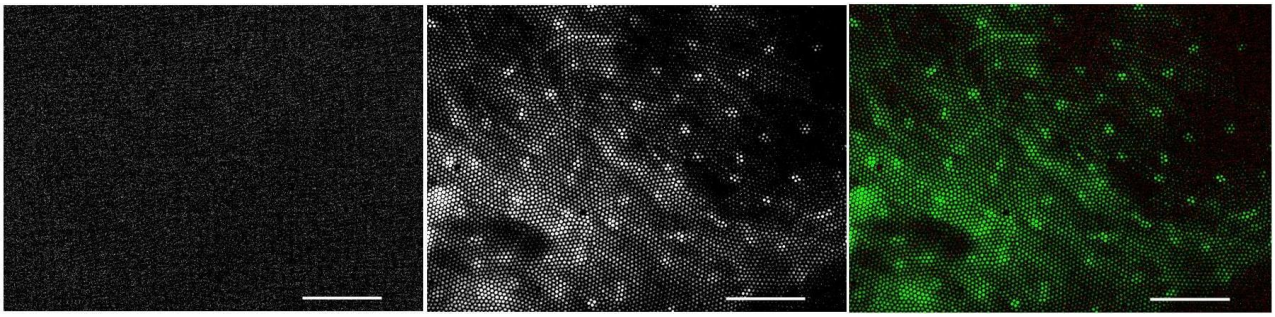

Figure 5. Microendoscope images of oral mucosa of a volunteer after application of proflavine solution only. Image of red channel (left), green channel (middle) and their composition (right). Scale bar is equivalent to 100 µm.

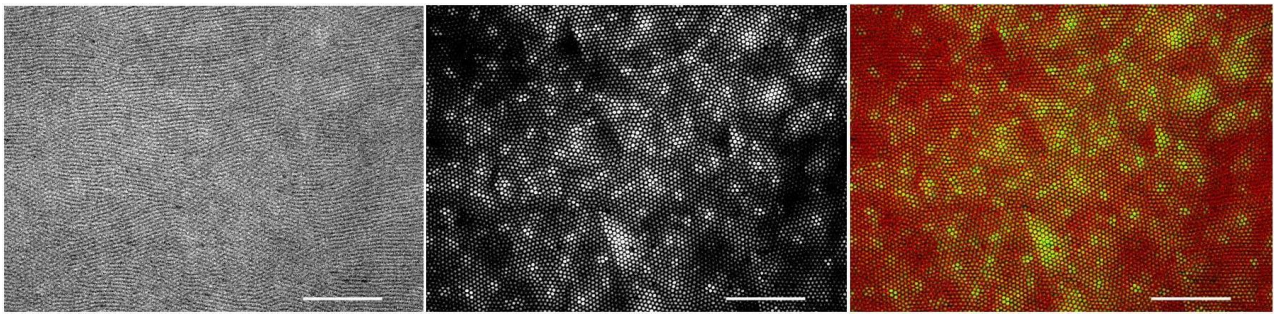

Figure 6. Microendoscope images of oral mucosa of a volunteer after ALA incubation and application of proflavine solution. Image of red channel (left), green channel (middle) and their composition (right). Scale bar is equivalent to 100 µm.

Figure 5 shows a weak red fluorescence background of the imaged tissue and figure 6 confirms the increase of this porphyrin emission after ALA incubation.
The presented results demonstrate the system competence for the simultaneous imaging of proflavine and PpIX. The outcomes suggest the possibility to detect abnormal tissue based on the nucleus morphology (proflavine emission) and in the amount of PpIX (red fluorescence). The dual-channel microendoscope can also be used for monitoring a PDT session mainly to see the PpIX formation and consuming during the illumination procedure. Which is valuable to determine, in a microscopic point-of-view, the best drug-light interval and the best illumination time for PDT. The thin probe facilitates the use of the system simultaneously with others equipment.

4. CONCLUSION

In this paper, the complete assembly and characterization of a dual-channel fluorescence microendoscope with subcellular resolution was presented. The system can be used to detect the presence of abnormal cells based on the microscopy fluorescence visualization of the cell nuclei and in the amount of PpIX. Also, the PpIX fluorescence can be useful to perform a better PDT dosimetry.

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