Time-resolved fluorescence spectroscopy for clinical diagnosis of actinic cheilitis

ALESSANDRO COSCI,1,2,3,5 MARCELO SAI TO NOGUEIRA,3,6 SEBASTIÃO PRATAVIEIRA,3 ADEMAR TAKAHAMA JR.,4 REBECA DE SOUZA AZEVE DO,4 AND CRISTINA KURACHI3

1Museo Storico della Fisica e Centro Studi e Ricerche Enrico Fermi, Piazza del Viminale 1, 00184 Rome, Italy
2IFAC-CNR, Istituto di Fisica Applicata “Nello Carrara,” Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Italy
3São Carlos Institute of Physics, University of São Paulo, PO Box 369, 13560-970, São Carlos, SP Brazil
4Faculdade de Odontologia de Nova Friburgo, Universidade Federal Fluminense, RJ, Brazil
5a.cosci@ifac.cnr.it
6marcelosaitonogueira@gmail.com

Abstract: Actinic cheilitis is a potentially malignant disorder of the lips. Its first cause is believed to be UV sun radiation. The lesion is highly heterogeneous, making the choice of area to be biopsied difficult. This study exploits the capabilities of time-resolved fluorescence spectroscopy for the identification of the most representative area to be biopsied. A preliminary study was performed on fourteen patients. A classification algorithm was used on data acquired on nine different biopsies. The algorithm discriminated between absent, mild, and moderate dysplasia with a sensitivity of 92.9%, 90.0%, and 80.0%, respectively. The false positive rate for healthy tissue (specificity) was 88.8%.

OCIS codes: (300.6500) Spectroscopy, time-resolved; (170.3650) Lifetime-based sensing; (170.3890) Medical optics instrumentation; (170.6510) Spectroscopy, tissue diagnostics; (170.6935) Tissue characterization.

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1. Introduction

Actinic cheilitis (AC) is a disease caused by prolonged and cumulative sun exposure that mainly affects the vermillion border of the lower lip. It mainly occurs in middle-aged fair-skinned people, which are often exposed to ultraviolet radiation (UV) due to outdoor working [1–3]. Equatorial and tropical latitudes can also play an important role in the incidence. AC is considered a potentially malignant disorder, since it may evolve into epithelial dysplasia in the form of squamous cell carcinoma (SCC) [4,5]. On a clinical and histological point of view, AC is highly heterogeneous, presenting either hyperplasia or atrophy, elastosis, cracks, and ulcerations. Currently, the golden standard for AC diagnosis is visual inspection followed by biopsy and histology. In this context, its high inhomogeneity makes difficult the selection of the biopsy area, thus resulting in potential misdiagnosis.

In the last two decades, new optical methods using the fluorescence properties of the tissues to assist clinicians in their inspections are emerging. Different spectroscopic features can arise from different tissue composition and architecture [6,7]. In a previous study, we evaluated the usefulness of tissue autofluorescence imaging in actinic cheilitis diagnosis. We found that wide-field fluorescence imaging enhances the heterogeneity properties of AC and, therefore, its diagnosis. Furthermore, by using an automated algorithm, it was possible to detect AC with sensitivity and specificity of 86% and 89.1%, respectively.

Unfortunately, fluorescence spectroscopy does not usually present high discrimination sensitivity, especially due to fluorescence emission overlapping among different molecules, and also the unspecific response related to inflammatory conditions. Time-resolved fluorescence spectroscopy (TRFS) may overcome this issue, since molecules with similar emission spectra can often be discriminated measuring their emission lifetimes [9–14]. Fluorescence alterations in lesioned tissue can arise from both metabolic and morphological anomalies. In the former an important role is played by two molecules, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) through the so-called Warburg Effect [15]. Both NADH and FAD show different lifetimes depending on their states (bound or free) [16,17]. The latter can be observed from the signal arising from collagen and elastin within the dermis. Indeed, cell infiltration due to cancer progression affects the collagen links in the stroma. Therefore, a lack of signal, or of the lifetime fingerprint, arising from collagen may be considered a signal of tissue lesion [18,19].

In the case of metabolic alteration of tissues, an important role is played by two molecules, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) through the so-called Warburg Effect [15]. Both NADH and FAD show different lifetimes depending on their states (bound or free) [16,17]. Furthermore, more information about the tissue histology can be obtained from the signal arising from collagen and elastin within the dermis. Indeed, cell infiltration due to cancer progression affects the collagen links in the stroma. Therefore, a lack of signal arising from collagen may be considered a signal of tissue lesion [18,19].

In order to have a feasible system for clinical spectroscopy investigation, a fiber-bundle-based TRFS device was employed. Fiber-bundle spectroscopy systems are well suited for skin and oral investigation, due to their feasibility. They have already been used for melanoma [20], basal cell carcinoma [21], in situ oral carcinoma [22] and skin photoaging [23]. Small fiber dimension allows to use them also for non-invasive inspection in hollow core organs as colon [24], thyroid [25], or vascular systems [26,27]. The satisfactory
sensitivity and specificity values achieved for discrimination of diseased tissue have promoted the use of these systems to assist medical doctor during surgeries, such as in the case of the brain [28] and oral cancer [22].

The system proposed in this study focuses on the determination of the metabolic state of cells within the tissue by TRFS. The two main molecules involved in these pathways are NADH and FAD that may be excited around 350 nm and 450 nm [29], respectively. The same wavelengths are also useful to detect alterations in the connective tissue, since they could also excite elastin and collagen. This study involved fourteen volunteers, ten patients with confirmed AC (measurements on nine biopsied areas) and four clinically normal volunteers as control. For each subject, fourteen fluorescence measurements in the lower lip were acquired. Each intensity profile was fitted by a bi-exponential decay, in order to discriminate between bound or free NADH and FAD. Further fluorescence decays were acquired on each single area that was selected for the biopsy excision. As well as the clinical features, the decays showed high heterogeneity in parameters obtained with the fit. Since none of the single parameters evaluated was found to be suitable for AC diagnosis, a k-nearest neighbors (KNN) classification algorithm was performed on all parameters in a multidimensional space, by using only the data arising from the biopsied areas and from the healthy volunteers lips. As a final result, it was possible to discriminate between healthy, mild and moderate dysplasia groups with a sensitivity of 92.9%, 90.0%, and 80.0%, respectively.

2. Materials and methods

2.1 Subjects

Fourteen subjects were involved in this study, ten patients with AC (age range 37 – 82 years old, 5 females and 5 males) and four as control (age range 48 – 71 years old, 2 females and 2 males). AC was confirmed on thirteen biopsied areas of lip of ten patients through histopathological investigation. Among these areas, nine were closely investigated using fluorescence lifetime measurements (six presenting mild epithelial dysplasia and three, moderate dysplasia). This study was accomplished with the approval of the Research Ethics Committee (Medical School, Antonio Pedro School Hospital, Fluminense Federal University).

2.2 Fluorescence lifetime spectroscopy set-up

Two different diode lasers emitting at 378 nm and 445 nm were used in order to excite mainly NADH and FAD, respectively (BDL-375-SMC and BDL-445-SMC, Becker and Hickl, Berlin, Germany). Lasers were used at the pulsed regime with a repetition rate of 80 MHz and a pulse duration of 50-100 ps. Excitation light was coupled in a bifurcated fiber bundle (BIF400-UV-VIS, Ocean Optics, Dunedin, Florida, USA) and delivered to the tissue. The common end of this bundle has two 400-µm-diameter fibers, positioned side-by-side. Fluorescence photons were collected by the fiber bundle and sent to a high-speed hybrid PMT detector (HPM-100-50, Becker and Hickl, Berlin, Germany). Two different bandpass filters at (440 ± 20) nm and (514 ± 15) nm were placed in front of the detector in order to mainly detection of NADH and FAD emission regions, respectively. The output signal of the hybrid PMT was read using a Time-Correlated Single Photon Counting (TCSPC) board (SPC-150, Becker and Hickl, Berlin, Germany). More detailed information is published elsewhere [20, 30].

2.3 Fluorescence lifetime measurements on patients’ lips

All fluorescence decay signals were acquired by the SPCM software (Becker and Hickl, Berlin, Germany). For each subject, 14 measurement sites in the lower lip were investigated (Fig. 1). Measurements were taken for each excitation wavelength. The used integration time was of 2 seconds, in all cases.
Fig. 1. Illustration of lower lip with the 14 measurement sites. For each point the integration time was of 2 s. In red is shown a zoom of the biopsied area (for example on site number 6) and the five collection points for lifetime investigation (indicated by the letters “a,b,c,d,e”).

For each patient with suspected AC, a fresh biopsy was excised for histopathological evaluation. Before the sample was excised, five fluorescence decays were acquired for each excitation wavelength, and in five different points of the area to be investigated by histology. The location for the point collection was chosen so that the five sites were similarly distant from each other.

2.4 Biopsies and histology

Biopsies using a 5 mm punch biopsy were performed in all patients with AC. The specimens were taken from areas optically characterized as fluorescence loss visualized by a widefield fluorescence system, in accordance with clinical features of white patches, red spots, erosions, crusts, ulcer, and induration. Tissue specimens identified by clinical and/or fluorescence indications were processed for histopathological analysis. Hematoxylin and eosin stained slides were evaluated by three oral pathologists to score epithelial dysplasia grade as mild, moderate or severe according to the World Health Organization criteria and classification [31]. All three pathologists reviewed all cases, performing their assessments independently of one another. The definitive dysplasia grade of the cases was reached by consensus assessment.

2.5 Data processing–lifetime measurements

Fluorescence decay signals were processed by using a homemade MATLAB routine. Briefly, the software performs a fitting of the experimental fluorescence decay $F(t)$ Eq. (1) to the convolution between the instrument response function (IRF, about 230 ps of full-width at half-maximum) with a bi-exponential decay:

$$F(t) = IRF * A * (a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2})$$ (1)

where $A$ is the amplitude, $a_1$ and $a_2$ are, for this study, normalized weights ($a_1 + a_2 = 100\%$), $\tau_1$ and $\tau_2$ are the corresponding lifetimes. The first set of data ($a_1, \tau_1$) corresponds to the faster lifetime component, while the latter ($a_2, \tau_2$) indicates the slower one. According to [17], for NADH, the faster component corresponds to its free state, while the slower one to its bound state. The opposite behavior is observed for FAD, where the faster and slower components
are related to its bound and free state, respectively. For each sample, 8 different parameters were obtained. All acquired parameters are shown individually on a boxplot figure. To assess whether exists a statistical difference between the groups, a Wilcoxon rank sum test (p < 0.01) was applied to each pair of groups considering the Bonferroni correction for multiple comparisons.

2.6 Data processing–k-nearest-neighbors classification of biopsies

To evaluate the efficacy of the fluorescence lifetime spectroscopy on the diagnosis of the actinic cheilitis, a k-nearest neighbors (KNN) algorithm was performed in order to classify each biopsy as normal tissue, mild and moderate dysplasia.

All the parameters acquired from the biopsies were placed in an 8-dimensional space, and the KNN algorithm [32,33], with k = 5 (5 neighbors), was used for classification by a 10-fold cross-validation method. The algorithm randomly chooses 10% of all the input points (observations) and uses these points as unknown samples to classify. The remaining 90% of the points are then used as a model for classification. Every point corresponding to an unknown sample is classified depending on its 5 nearest neighbors. If most of them are normal tissue, it will be classified as normal. The WEKA software was used to perform the KNN algorithm analysis [32,34].

3. Results

3.1 Fluorescence lifetime measurements on patients’ lips

Figure 2 presents the data distribution of bi-exponential parameters evaluated by TRFS through a boxplot visualization. Normal tissue group stands for all the points collected in the healthy volunteers’ lips, and dysplastic tissue group are all the points collected from the whole lip of patients with confirmed AC. The other groups represent observations of biopsied areas classified as mild and moderate dysplasia after histopathological investigation. A high variability was observed for the whole lip measurements. For some parameters, the heterogeneity of measurements for biopsied areas are comparable to that for the whole lip.

The boxplots show the following trend in the evaluated parameters:

- \( a_{1,378}, \tau_{2,378} \) tend to be higher, and \( \tau_{1,378} \) lower, where \( a_{n,\lambda} \) and \( \tau_{n,\lambda} \) are, respectively, the percentage weight or lifetime related to the fast (\( n = 1 \)) or slow (\( n = 2 \)) lifetime component, and to an excitation wavelength (378 nm or 445 nm), for normal tissue when it is compared to other groups;

- \( \tau_{1,445}, \tau_{2,445} \) tend to be higher, and \( a_{1,445} \) lower for biopsied regions with confirmed mild dysplasia when comparing them to those with moderate dysplasia;

- \( a_{1,445} \) and \( \tau_{2,445} \) tend to be higher for the normal tissue when it is compared to the dysplastic tissue group;

- \( \tau_{1,445} \) tend to be lower for normal tissue compared to the mild dysplasia group.
Fig. 2. Boxplots for percentage weights and fluorescence lifetimes using 378 nm and 445 nm excitations. Black and red boxplots present the data distribution for 14 investigated areas from healthy volunteers and AC patients, respectively. Blue and purple boxplots present the same distribution for biopsied areas classified by histopathological assessment as mild and moderate dysplasia areas. Significant statistical different groups (Wilcoxon Rank Sum Test with Bonferroni correction, p < 0.01) are marked using the star symbol (*).

3.2 Classification of biopsied areas through KNN algorithm and ten-fold cross-validation

To test the discrimination efficacy of the data points as belonging to normal or dysplastic tissue groups, the KNN algorithm with K = 5 was chosen. The choice of a simple algorithm as KNN was performed in order to avoid any possible overfitting from more complex algorithms. Since this study it is at a preliminary stage, a ten-fold cross-validation method was used to create the points to be used as the training and test sets. The algorithm scored the test set by evaluating each point proximity to its 5 closest neighbors. Using all the decay
parameters evaluated exciting at both wavelength and a KNN classification algorithm, it was possible to discriminate between normal and dysplastic tissue with a sensitivity and a specificity of 67.9% and 90.8%, respectively.

The low sensitivity achieved is probably a result of the high heterogeneity of the lips presenting AC. With dysplastic tissue were considered all the 14 points of the lip investigated. While some of the points were confirmed as AC by histopathological assessment, some regions could not show any dysplasia and therefore could have the same features of healthy tissues. Certainly, the main aim of this study is the evaluation of the best area to be biopsied by means of TRFS.

Therefore, on a second stage, only the points interrogated at the biopsied areas were compared with the data acquired from healthy volunteers. Once the KNN classification algorithm was performed on the whole parameters multidimensional space, healthy, mild and moderate dysplasia groups were classified as reported in Table 1. The histopathological diagnosis is shown on the left hand column and results for classification using TRFS measurements are presented in the top row.

| Classified as healthy by KNN algorithm | Classified as mild dysplasia by KNN algorithm | Classified as moderate dysplasia by KNN algorithm |
|----------------------------------------|---------------------------------------------|-----------------------------------------------|
| Classified as healthy by histopathology | 52                                          | 3                                            |
| Classified as mild dysplasia by histopathology | 3                                           | 27                                           |
| Classified as moderate dysplasia by histopathology | 2                                           | 1                                            |
| Classification results                  | Classification results                      | Classification results                        |
| Classified as healthy by histopathology | 52                                          | 3                                            |
| Classified as mild dysplasia by histopathology | 3                                           | 27                                           |
| Classified as moderate dysplasia by histopathology | 2                                           | 1                                            |

As a final result, it was obtained a sensitivity of 92.9%, 90.0%, and 80.0%, for healthy, mild and moderate dysplasia, respectively. Specificity in assessing healthy tissue was 88.8%.

4. Discussion

Actinic cheilitis is a highly heterogeneous lesion of the lower lip, due to prolonged sun exposure. Its histological appearance could vary from atrophy to elastosis, usually together with hyperplasia, cracks, and ulcerations. Therefore, it is reasonable to expect that also the fluorescence arising from its biochemical composition should present high heterogeneity. Indeed, it was not possible to discriminate AC from healthy lips by means of a single parameter out of the fluorescence lifetime measurements. Since AC could affect only a small area of the lip, any attempt to classify the TRFS results could be accomplished only at the biopsied areas. As a reference for healthy tissue, it was possible to take into account not only the areas in which the dysplasia was absent, but also all the 14 points measured for each control volunteer. Heterogeneity in the case of normal tissue could arise from different conditions in the lip area as dry and keratinized tissue, vessels and inflammation. Further analysis should involve the correlation with clinical features and investigated region of the lip.

The detected fluorescence arises from the contribution of all fluorophores within the investigated tissue volume. To interpret the obtained result, it is necessary to take into account not only the cell metabolic pathways, but also the morphological structure. Two studies by Pavlova et al. [18,19] demonstrated that, in healthy tissues, most of the fluorescence signal is originated by the connective tissue underneath the epithelium. Fluorescence loss due to cell infiltration inside the stroma was found also on visual fluorescence investigation on oral tissue [34,35]. Furthermore, sun exposure was found to promote an increase of elastin composition into the dermis and a consequent collagen regression [36].
and $a_{1,445}$ could be related to collagen abundance, therefore to a healthy tissue [37]. Higher values of $\tau_{1,378}$ for dysplastic tissues can instead be originated from a higher presence of bound NADH, although a possible contribution from collagen needs to be taken into account in this case. When using the 378 nm excitation, the whole scenario may be more complicated, since an important role could be played by NADPH that could be involved in different biosynthetic pathways. Those pathways can be related to different cellular pathologies [38]. Furthermore, the evaluated value for $\tau_{1,378}$ when fitting the experimental decay by a bi-exponential curve was found to be in the order of 1 ns. This value, usually associated to free NADH, could also include contributions from bound NADH, as it is presented in [39]. When exciting at 445 nm, lower values of $\tau_{1,445}$ in healthy tissue may arise from the contribution of bound FAD, since this molecule is presented only on the oxidative phosphorylation [17]. These tendencies can be observed at the boxplots, but still data distribution did not allow to discriminate between healthy and dysplastic tissue. Finally, by looking carefully to the whole situation and using a multidimensional space of the evaluated parameters, it was possible to identify two different clusters: one for AC and another for healthy tissue. The KNN algorithm is a simple algorithm that classifies each unknown point by means of its k proximal neighbours. The use of a simple algorithm in the preliminary stage of our study is useful to avoid overfitting effects that can be introduced by the use of more complex algorithms.

5. Conclusion

Actinic cheilitis is a disorder of the lip that can potentially progress to cancer [4,5]. The tissue degeneration occurs mainly in the epithelium and can, consequently, further infiltrate into the stroma. Fluorescence imaging and spectroscopy are valid tools for evaluating cell metabolism and collagen concentration. These techniques were already found useful for AC assessing during clinical evaluation [8], but they still do not offer a valid method for the selection of the area to be biopsied.

The aim of this study was to evaluate the potential of TRFS for assessing the best area for the punch biopsy. Twenty different points on healthy volunteer and suspected AC were acquired, fourteen in the lower lip and six on the upper one. Further five points were acquired in the area punched for the biopsy.

Fluorescence decays were measured at two different excitation wavelengths, 378 nm and 445 nm. Those wavelengths are particularly useful for detecting metabolic alterations and differences in tissue composition. The former could be observed by a different abundance of NADH and FAD, and their relative presence in a free or in a bound state. In order to focus mainly in the signal arising from those molecules two filters with detection bands of $(440 \pm 20)$ nm and $(514 \pm 15)$ nm were used. The latter is characterized by a lack of signal coming from collagen. This could be originated by cell infiltration inside the dermis or by sun induced elastosis. All the fluorescence decay curves were fitted by a bi-exponential decay. The first representation of fitted parameters was shown by means of boxplot graphs. Although a tendency for the parameters could be observed for each histopathological result, the data variance does not allow any classification algorithm using only one parameter.

All the fluorescence decay curves were fitted by a bi-exponential decay. The first representation of fitted parameters was shown by means of boxplot graphs. Although a tendency for the parameters could be observed for each histopathological result, the data variance does not allow any classification algorithm using only one parameter.

A KNN algorithm was applied using five nearest neighbors by means of ten-fold cross validation methods. Using this technique, it was possible to assess healthy, mild and moderate dysplasia with a sensitivity of 92.9%, 90.0%, and 80.0%, respectively. In addition, the specificity in finding healthy tissue was 88.8%. Then, TRFS was found to be a promising tool for the selection of the area to be punched for biopsy. This method can be further improved by involving more patients and by using of more complex algorithms.
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