Autofluorescence spectroscopy for the classification of oral lesions

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Chapter 1

Introduction

1.1 Introduction

Oral cancer is a disease that occurs in approximately 800 new patients in the Netherlands each year. Most of these patients have a history of heavy smoking, often in combination with the frequent use of alcoholic drinks, especially strong liquors. More than 90% of these cancer patients suffer from oral squamous cell carcinomas which arise from the upper layer of the tissue: the oral mucosa. It is this mucosa that has been exposed to tobacco and alcohol, and is therefore at risk for developing premalignant lesions and invasive tumours. During the process of so-called 'field cancerization', multiple areas of the oral mucosa undergo carcinogenic changes. This is an important feature of oral carcinogenesis and it can explain why the occurrence of second primary tumours in patients is so often seen. In fact, 28% of the patients diagnosed with a squamous cell carcinoma (SCC) of the oral mucosa will develop a second primary tumour within ten years. As with all cancers, the prospects for the patient are better when the malignancy is found in an early stage. Treatment of small tumours, without regional metastases, give higher survival rates, better functional and esthetic results and a lower morbidity than in the case of advanced tumours. Early treatment strongly improves the survival rates and results in a lower morbidity. For example, a premalignant lesion can be removed by CO_2 laser evaporation of the affected epithelium (a mildly invasive treatment), while advanced squamous cell carcinoma's generally require extensive surgery and/or radiotherapy which may profoundly affect certain essential functions like swallowing and speech. Furthermore, these advanced carcinomas can metastasise, which significantly reduces the survival chances. For this reason, patients who are at high risk for developing oral cancer, i.e. patients that have been treated for oral cancer, are submitted to a strict screening protocol by a specialized oral and maxillofacial surgeon (oral oncologist). Upon discovery of a lesion of the oral mucosa by visual inspection, it is impossible to classify the lesions as harmless or potentially malignant. Most premalignant lesions present as whitish (leukoplakia) or reddish (erythroplakia) lesions and usually resemble some far more common benign lesions, even to the experienced eye. Only a biopsy can provide the final diagnosis. Unfortunately, the number of biopsies that can be obtained within one session is usually limited due to the complex anatomy of the head and neck region. Furthermore, the biopsy can result in some discomfort for the patient and of course the pathological examination costs time and money. Another important drawback is that the biopsy results are not always representative for the complete lesion. An oral lesion can contain local premalignant changes at one position, while it can still be benign at a position only a few millimetres away. This can result in underdiagnosis, or the need for repeated biopsies if the oral oncologist is not convinced that a lesion is benign.

1.2 Optical spectroscopy

The disadvantages of the traditional biopsy form a strong clinical rationale for developing other techniques to improve the detection of early premalignant changes in the oral mucosa. One of these techniques under investigation is optical spectroscopy. Optical spectroscopy allows non-invasive physical and chemical characterisation of biological tissues. The structural and chemical composition of cells and tissues strongly influences their optical features, and therefore alterations in the optical characteristics may indicate the presence of diseased tissue. Optical spectroscopy may provide possibilities in the early detection of cancerous tissues in humans. Biochemical and structural/morphological information can be gained by measuring absorption, (auto)fluorescence, elastic scattering or Raman scattering. These spectroscopic techniques each have separate physical bases and all have the potential to become an adjuvant method to conventional cancer detection methods. However, of all these optical techniques, spectroscopy of tissue autofluorescence has

been favoured the most promising. In a pilot study, we found excellent results for distinguishing cancerous tissue from healthy oral mucosa. Also, the techniques required for autofluorescence spectroscopy are relatively easily applicable and cheap. Therefore, we decided to investigate the value of autofluorescence spectroscopy in a clinical study. Such a study requires extensive analysis of a wide variety of lesions and tissue conditions and we have therefore limited ourselves to solely investigate autofluorescence spectroscopy as a technique for cancer detection.

1.3 Autofluorescence

Autofluorescence is the fluorescence of tissues to which no chemical substances have been applied: it is the natural fluorescence of the tissue itself ('auto'). Fluorescence in general is the process by which excitation with light evokes the emission of light of a different (lower) wavelength, i.e. red-shifted light. In this thesis, the excitation light is in the near-UV to visible range and the evoked autofluorescence is in the visible to near-infrared range. Fluorescence is the result of a process that occurs in certain molecules called fluorophores. The process responsible for the fluorescence of fluorophores is illustrated by the electronic-state diagram (Jablonski diagram) shown in Figure 1.1.

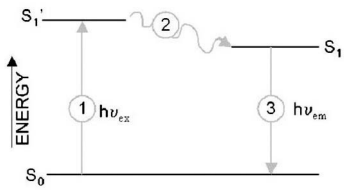

Figure 1.1 Jablonski diagram.

(1) : A photon of energy $h\nu_{ex}$ (h=Planck's constant, ν_{ex} =excitation wavelength) is supplied by an external source such as a lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1').
(2) : The excited state exists for a short time (picoseconds) during which the energy of S_1' is partially dissipated through internal conversion, yielding a relaxed singlet excited state (S_1) that typically exists for 1-10 nanoseconds and from which fluorescence emission originates. Not all the molecules initially excited by absorption return to the ground state (S_0) by fluorescence emission; other processes may also depopulate S_1. The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted to the number of photons absorbed, is a measure of the relative extent to which these other processes occur.
(3): A photon of energy $h\nu_{em}$, with $h\nu_{em}$ the emission wavelength, is emitted. The fluorophore returns to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower and therefore of longer wavelength than the excitation photon $h\nu_{ex}$. The difference in energy or wavelength represented by ($h\nu_{ex} - h\nu_{em}$) is called the Stokes shift. The difference in wavelength between excitation and emission light allows one to easily separate the evoked fluorescence light from the reflected excitation light, which is generally much higher in intensity. Continuous excitation of the fluorophore causes continuous emission of fluorescent light, unless the fluorophore can be destroyed by excitation ('photobleaching').

1.4 Biofluorophores

In the literature, various naturally occurring biofluorophores such as keratin, porphyrins, NAD(P)H, collagen and elastin have been claimed to be useful for the classification of lesions. Carcinogenic processes produce alterations at the cellular level but also in the structural tissue composition. Both may be reflected in autofluorescence spectral shape and intensity. For example keratin, which is located mainly at the upper layer of the mucosa, is strongly fluorescing. For this reason, hyperkeratotic lesions will show more keratin autofluorescence and possibly can be distinguished from healthy mucosa thanks to this. Another example is porphyrin-like fluorescence, which has been claimed to be associated with tissue alterations and with malignancy in particular. If this is true, the relatively narrow porphyrin fluorescence peak may be informative about the mucosa under investigation. For distinguishing between different lesion types, in particular between (pre-)malignant and benign lesions, much attention has been given to NAD(P)H, i.e., the reduced coenzyme nicotinamide adenine dinucleotide and its phosphorylated form. Having both oxidized NAD(P)+ and reduced NAD(P)H forms, coenzymes NAD(P) play an important role in cellular metabolism. Only the reduced form is fluorescing. Since (pre-)malignant lesions are characterized by cell proliferation, which costs a lot of energy and causes the metabolic activity to increase, the reduced form of NAD(P)H is expected to predominate in these lesions. A higher observed autofluorescence intensity may result. However, no convincing evidence for this hypothesis has been given yet.

Collagen and elastin from the connective tissue layer can be decomposed by processes associated with disease, in particular for (pre-)malignant lesions. This may be reflected in a lower autofluorescence intensity. Epithelial thickening may shield the collagen and elastin fluorescence from the deeper layers. Also, angiogenesis may result in an increased amount of blood, which contains hemoglobin that will partly absorb both excitation and fluorescence light. In this way, the presence of disease will be reflected in the autofluorescence spectra in a less direct way. From this last example, it is clear that blood absorption can be useful on its own for lesion characterization. The same goes for scattering, which may for example be increased in malignant tumours because of the large number of scattering nuclei in cancerous mucosa.

1.5 Classification

The classification of lesions will probably be difficult. Due to the optical properties of living tissue, it is impossible to retrieve the exact fluorophore concentrations in the oral mucosa by analyzing autofluorescence spectra. For example, hemoglobin in the blood will partly absorb the excitation light as well as the evoked autofluorescence. Since the distribution of blood within the tissue under investigation is not known, and because the unknown variable oxygenation influences the wavelength-dependency of blood absorption, these effects cannot be undone mathematically. Furthermore, tissue contains many light scattering particles, such as cell nuclei and organelles. The scattering effects that these induce are wavelength-dependent and therefore do not only change the total autofluorescence intensity by multiple reflections within the tissue, but also the spectral shape. Since the distribution of scatterers within the tissue is not known, these artifacts cannot be completely removed from the autofluorescence spectra. Next to these scattering and absorption effects, most fluorophores have very broad and overlapping emission peaks that complicate the determination of fluorophore concentrations.

Since the exact concentrations of fluorophores cannot be reliably calculated from the spectra, it will not be possible to construct a simple lesion classification rule. Other, more empirical mathematical approaches have to be applied to extract the relevant tissue characteristics. For this reason,

various techniques have been applied in the literature, such as principal components analysis, emission wavelength ratios and artificial neural networks.

1.6 Aim of our study

Several scientific papers indicate that spectroscopy of tissue autofluorescence provides a sensitive and easily applicable method for detection of small and superficial lesions. Published data show that this technique may be able to differentiate between normal mucosa, hyperkeratosis and (pre)malignant lesions of the mucosa at an early stage. In this thesis, we will study the applicability of autofluorescence spectroscopy in the diagnosis of oral (pre)malignant lesions. Our hypothesis is that autofluorescence spectroscopy will detect premalignant lesions and squamous cell carcinoma of the oral cavity at an earlier stage than visual inspection. We will try to establish the usefulness of autofluorescence spectroscopy as a guide to decide which location is best to take biopsies and as a method to detect (pre-)malignant lesions at an earlier stage. We need to know whether the method is capable of distinguishing between benign lesions on the one hand, and dysplastic and cancerous lesions on the other hand. If this distinction can successfully be made, we will study which alterations in the autofluorescence emission spectrum are responsible for the classification.

In this thesis we study:

- **Influences of anatomical location on the autofluorescence characteristics of oral mucosa**. The oral cavity is lined with a rich variety of mucosal types. The resulting influences on autofluorescence characteristics might be large and possibly have to be taken into account when performing lesion diagnostics.
- **Influences of individual characteristics on autofluorescence characteristics of the oral mucosa**. For example, tobacco is known to produce tissue alterations that may eventually lead to cancer. It is conceivable that the influence of smoking is also reflected in autofluorescence of healthy oral mucosa. The same goes for the influence of alcohol consumption. We also examine influences of age, gender, wearing of dental prostheses and skin colour.
- **Lesion diagnostics with autofluorescence spectroscopy.** We use various combinations of classification algorithms and excitation wavelengths. Three clinical questions are addressed: oral cancer vs. healthy mucosa, lesions vs. healthy mucosa, and (pre-)malignant vs. benign lesions.
- **Autofluorescence microscopy of freeze-dried tissue sections from lesions**. We investigate the autofluorescence characteristics of the different tissue layers. A comparison with Raman spectra from the same sections is made.
- **Reflectance spectra in lesion diagnostics.** These white light spectra, which are sensitive to absorption and scattering only, are recorded in addition to the autofluorescence spectra in order to study their potential for lesion diagnostics, combined with autofluorescence or on its own; and to investigate to a first approximation the influence of blood absorption and tissue scattering effects on the diagnostic potential of autofluorescence spectroscopy.
- **Scientific literature.** To put our own results into a perspective, we extensively review the literature on autofluorescence imaging and spectroscopy for the oral mucosa.