CHAPTER 39

Optical diagnosis of cancer and potentially malignant lesions

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Introduction

In the medical sciences, diagnosis refers to the *knowledge* and *discrimination* of the characteristics of the pathology or general condition of a patient. Diagnosis is also considered the procedures used to obtain the information for the identification of the pathology or the metabolism of the tissue. To achieve the diagnosis of a lesion or of the patient's clinical status, the health professional obtains information through anamnesis, clinical examination, and laboratory tests.

In dentistry, the gold standard for diagnosis of cancer, potentially malignant disorders, and other pathologies that affect soft tissues is biopsy and histopathological examination under an optical microscope. Biopsy is indicated only when the dentist suspects, based on the clinical appearance, that the lesion is potentially a carcinoma or severe dysplasia. Depending on the lesion size and its anatomical site, the clinician chooses between a complete excision, mainly for small lesions, and an incisional biopsy. In the latter, a tissue sample is excised and the material sent to a pathology laboratory. Both tissue materials, the whole lesion or the sample, are processed and analyzed under an optical microscope by a certified pathologist. The pathologist will give the diagnosis based on the characteristics seen, especially cell morphology and tissue architecture.

Clinical examination involves palpation inspection and visual identification of lesion characteristics such as color, texture, macroscopic morphology, and surface homogeneity, which must always be compared to the features of the normal tissue. These conventional diagnostic procedures for oral cancer are highly dependent on the clinician's skills and experience in detecting malignant clinical features. Cancer diagnostics in the initial stages of cancer are not simple, since their clinical characteristics are similar to those of the more prevalent benign disorders. The discrimination between dysplasia and carcinoma *in situ* (CIS) is also a clinical challenge.

Another relevant issue is the determination of the biopsy site in a non-homogenous lesion. Distinct surface characteristics result from different histological features that may represent distinct pathological diagnoses. If the clinician does not choose the best site for incisional biopsy, a cancer may be misdiagnosed. It is not feasible to convince the patient to have several biopsies taken from a large lesion. The clinician must perform a detailed clinical diagnosis to enhance the probability of defining the dysplasia and malignant areas, if present.

Optical techniques have been proposed as auxiliary diagnostics tools to clinical examination. These techniques are based on the principle that *light–tissue interactions* change with cancer progression and the analysis of these optical phenomena gives information on biochemical and structural tissue composition. The conventional clinical examination is performed under white light and the re-emitted light visualized at the tissue surface is a result of reflectance, scattering, and absorption interactions in the illuminated region. If the illumination and detection conditions are modified and assembled to optimize the interrogation of some specific light–tissue interactions, some biomolecules and tissue architecture information can be targeted.[1]

Optical techniques are attractive tools since they provide an objective fast response through non-invasive and non-destructive procedures. In this way, the diagnosis is less subjective and dependent on the clinician's expertise.

Light–tissue interactions

When a biological tissue is illuminated, light–tissue interactions take place at the surface and within the tissue. These interactions are modified depending on the light parameters and tissue optical characteristics. The main light parameters that influence these phenomena are: wavelength, spectral bandwidth, and pulse width.[1,2] Biological tissues are composed of several molecules that alter the photon pathways inside the tissue. As a result, the same wavelength will interact differently depending on the biochemical composition and tissue architecture, and the same tissue will interact differently depending on the illumination parameters.

Lasers in Dentistry: Guide for Clinical Practice, First Edition. Edited by Patrícia M. de Freitas and Alyne Simões.

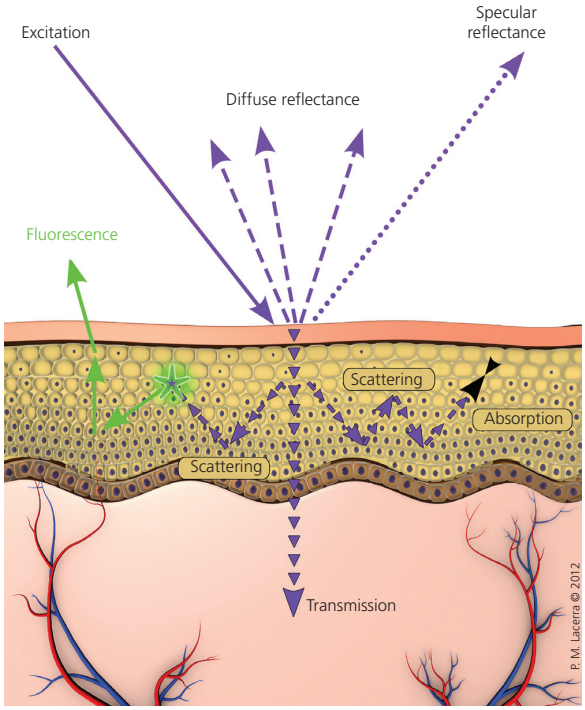


Figure 39.1 Schematic showing the light–tissue interactions taking place in the oral mucosa. The incident light (excitation) is delivered to the tissue surface and the transepithelial photon pathways are shown (diffuse and specular reflectance, scattering, fluorescence, absorption, and transmission (image created by P.M. Lacerra (2012). Provided by Kurachi).

Light–tissue interactions are classified into the following types (Fig. 39.1):

- Reflectance (specular and diffuse);
- Scattering;
- Absorption;
- Transmission;
- Luminescence (fluorescence and phosphorescence).

When photons are delivered to a tissue surface, some are reflected and do not actually interact with the biological molecules. The tissue surface acts as a mirror, reflecting the light without changing its characteristics. This phenomenon is called *specular reflectance* and does not give any information about the tissue. The photons that penetrate the tissue are the ones that provide diagnostic information.

Photons travelling within the tissue will change their direction when they meet a component with a different refractive index. Tissue layers of different biochemical composition and structure show distinct refractive indexes, and if an interface between these layers is in the path of a photon, it will modify the direction in which the photon travels. In oral mucosa, the keratin layer, epithelial tissue, and underlying lamina propria (fibroblasts, connective tissue, small capillaries, inflammatory cells, and extracellular matrix) have different refractive indexes. Photons travelling from the surface into the inner regions of the oral mucosa will undergo several changes in their direction, a light–tissue interaction known as *scattering*. This phenomenon also takes place at the microscopic level, with photons changing direction as they meet the cell membrane, nuclei, and organelles.

After several scattering interactions, some of the delivered photons will exit from the tissue surface. This light emerging at the tissue surface shows the same wavelength as the incident light, but differs in irradiance and direction. This re-emitted light after multiple scattering is called the *diffuse reflectance*.

Photons that are not absorbed and do not change their direction are transmitted through the tissue layers. When *transmission* occurs, the optical path is not modified by the tissue as the photons have not been influenced by the presence of the biological components.

Absorption is the process that happens when a biological component absorbs the photon energy. This energy can be used for a biochemical reaction or can result in molecular vibration (heat) or in photoemission (luminescence). For most available laser therapies, absorption is the most relevant light–tissue interaction that must take place for the tissue response, such as ablation, vaporization, and carbonization. The main biological absorbers are hemoglobin and melanin.

Some biological chromophores absorb the photon energy and later emit light, through a process called *luminescence*. This photoemission is one of the possible ways that an excited molecule can lose energy to return to its most stable ground electronic state. Luminescence is classified as fluorescence or phosphorescence, according to the length of time that this phenomenon occurs. Most biological luminescent molecules emit light through fluorescence, showing lifetimes in the nanosecond range.

Fluorescence for cancer diagnosis

In optical diagnostics, all these light–tissue interactions provide information about tissue biochemical composition and structure (architecture) and can be used in tissue discrimination. The great majority of the available optical diagnostic systems for dentistry are based on the detection of fluorescence because fluorescence is sensitive to the changes in biochemical composition that occur during malignant development, as well as in other pathological conditions. Structural changes that occur with cancer progression also modify the tissue fluorescence. As a result, the fluorescence emission from a normal oral mucosa is distinct from that from an oral cancer.

The most important biological fluorophores involved with malignant progression are coenzyme nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), collagen, elastin, and porphyrin. NADH and FAD are molecules involved in important metabolic reactions, and their relative concentrations provide information concerning the metabolic

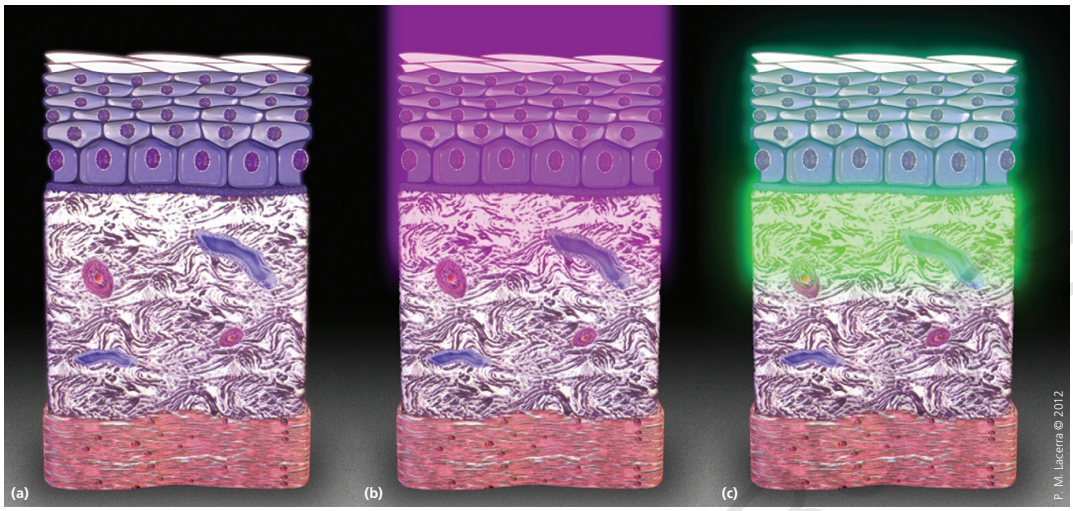


Figure 39.2 Schematic of the optical paths for fluorescence of normal mucosa: (a) Normal mucosa histology; (b) illumination with violet excitation; (c) fluorescence (image created by P.M. Lacerra (2012). Provided by Kurachi).

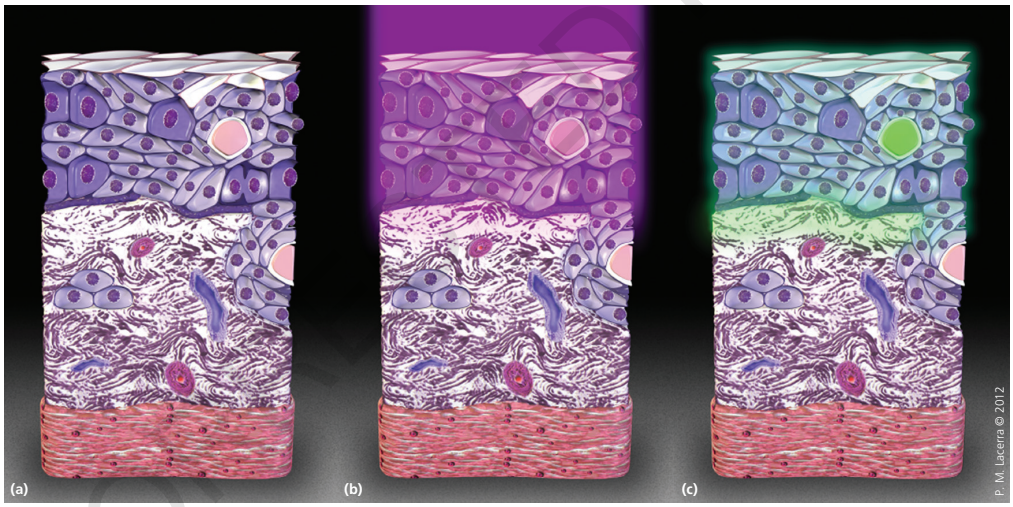


Figure 39.3 Schematic of the optical steps for fluorescence from oral carcinoma: (a) Carcinoma histology; (b) illumination with violet excitation; (c) fluorescence (image created by P.M. Lacerra (2012). Provided by Kurachi).

status of the tissue. Collagen and elastin are structural proteins that are destroyed during carcinoma invasion into deeper tissue layers. When these proteins are destroyed, they stop emitting fluorescence. Porphyrin has higher concentration in malignant cells compared to normal oral epithelium.

The most commonly observed fluorescence behavior related to cancer detection is decreased green emission, due to the diminished collagen emission around 520 nm, and increased red emission around 630 nm, as a result of a higher porphyrin concentration. The main analysis in reported studies of oral cancer is based on the red/green emission rate.

Biological molecules absorb light in the ultraviolet–violet spectrum with higher efficiency, so the available optical systems use light in the spectral region of 380–450 nm for tissue excitation.

Figures 39.2 and 39.3 show the optical paths taken occur at the tissue level for normal and cancer oral mucosa, respectively. Normal epithelium of the oral mucosa is composed of organized layers of epithelial cells, covered by keratin in the keratinized mucosa (Fig. 39.2a). If normal epithelium is illuminated at the surface with a violet light (Fig. 39.2b), all the light–tissue interactions described above take place. Some part of the energy is absorbed by the fluorophores in the optical path, and these

re-emit light as fluorescence. The most important fluorophores in the oral mucosa, when excited by light in the violet spectrum, emit light in the green spectrum (Fig. 39.2c). In epithelial cells, the main fluorophore that is excited by light in the violet spectrum is NADH, and in the stroma is collagen. Collagen is the molecule that mainly contributes for the final fluorescence by mucosal cells. The photons of the fluorescence must penetrate the tissue and can be absorbed or undergo multiple scattering before exiting the mucosal surface. Another important fluorophore in the oral mucosa is keratin, which emits an intense green fluorescence.

In comparison to the normal mucosa, oral carcinoma has a disorganized epithelium, increased number of cell layers, and disruption of the collagen cross-links (Fig. 39.3a). These tissue changes, after surface illumination with violet light (Fig. 39.3b), result in decreased green fluorescence (Fig. 39.3c). The increased number of layers enhances the tissue thickness and fewer excitation photons reach the stromal layer, so fewer collagen molecules are excited and emit fluorescence. As a result, fluorescence from a carcinoma shows decreased green fluorescence when compared to normal oral mucosa.

If the mucosa is covered by a thick layer of keratin, the fluorescence will be intense, but the epithelial and stromal layers will not be excited. A false-negative result for carcinoma detection using fluorescence can therefore result if the carcinoma lies beneath this thick keratin layer.

A decreased green fluorescence as a result of an inflammatory benign disorder may lead to a false-positive response in cancer diagnosis. In these inflammatory conditions, an increased microvasculature results in a higher concentration of hemoglobin, enhanced absorption of the excitation light, and increase in fluorescent photons. The final fluorescence collected at the tissue surface will be lower, but in this case is due to a higher absorption and not to decreased collagen fluorescence.

All these factors must be evaluated when using fluorescence diagnostics. No optical technique will be valid for diagnosis without an understanding of the affect of the histological and macroscopic tissue features on light–tissue interactions. The clinician must apply the optical techniques as an auxiliary tool to all the other usual procedures. The advantages of the optical techniques are their objectivity, fast response, as well as being non-invasive procedures.

Optical techniques can be classified into imaging and spectroscopy modalities. The first of these gives a result that can be at the macroscopic or microscopic levels.

Imaging techniques for diagnosis of oral cavity malignancies

Reflectance imaging

Imaging performed with conventional white light (WL) reflectance can be easily improved by selectively focusing on a single feature of the tissue. The images obtained are a superimposition of all the chromatic components of the light reflected by the surface of the sample and the light that has experienced multiple scattering and absorption deep inside the tissue. One way to selectively look at a single feature of the tissue is to use one specific wavelength, by placing a narrow band filter after the WL source emission and before the tissue illumination. For example, the absorption of a specific molecule, such as hemoglobin, can be targeted.[3] In this case, the clinical feature that will be selected is the tissue vasculature. This technique is usually referred to as *narrow band imaging* (NBI).

Another method uses polarized illumination and detection. When reflected from a surface, the polarity of the incident light is preserved, whereas when it is scattered by tissue this polarization is lost. Therefore, using polarized illumination and detection of the reflectance, it is possible to discriminate between light originating from the surface (specular reflectance) – by selectively detecting reflected photons that have the same polarization as the incident light, and light originating from the deeper layers of the tissue (diffuse reflectance) by selectively detecting photons with orthogonal polarization.[4] This technique is referred to as *polarization reflectance imaging*.

The main application of polarization reflectance imaging is to enhance the vessel pattern of the tissue under investigation. The vessel pattern plays an important role in revealing angiogenesis in suspicious areas and may help to discriminate cancer lesions from a false-positive response arising from inflammatory tissue, as has been demonstrated in bladder[5] and colon.[6] Lindeboom et al. used orthogonal polarization reflectance imaging (OPS) to characterize the microcirculatory changes in tongue squamous cell carcinoma (SCC) compared to the normal contralateral side.[7] Using orthogonal polarization detection, with respect to the incident light, the authors were able to achieve better visualization of the vessel pattern beneath the tissue surface. For SCC, dilated and disorganized vessels accompanied by hemorrhagic areas were observed, while the normal contralateral tissue showed no abnormal capillaries or any vessel disarrangement. In another study, Basiri et al. used polarized reflectance imaging to diagnose familial adenomatous polyposis by looking at the vasculature of the oral mucosa, with a sensitivity and specificity of 90.9% and 90.0%, respectively.[8]

Roblyer et al. developed a new set-up that can perform both imaging modalities, narrow band and polarization reflectance.[9,10] A simplified schematic of a system that combines OPS with NBI is shown in Figure 39.4. In their study, Roblyer et al. used OPS, NBI, and fluorescence imaging. Five different types of tissue were investigated: a normal labial mucosa of a healthy volunteer, a leukoplakia at the right gingiva, a leukoplakia at the tongue with confirmed moderate dysplasia, an erythroplakia of the tongue with confirmed severe dysplasia, and a cancer of the lateral tongue. The wavelengths chosen for NBI were 420, 430, 530, and 600 nm as these matched the hemoglobin absorption peaks. Using distinct excitation wavelengths, it was possible to interrogate different tissue depths, since the higher wavelengths penetrate tissue more deeply. Therefore, 420-nm (blue) light

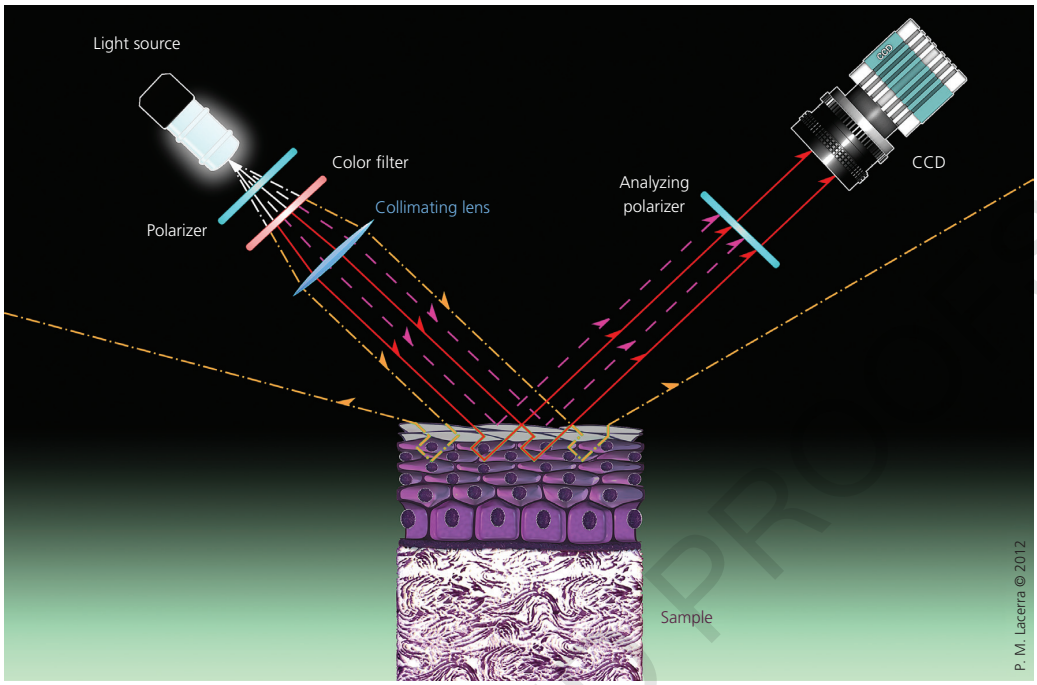


Figure 39.4 System combining OPS and NBI. White light is first polarized. A narrow band filter is used to select the wavelength range of illumination. Reflectance and scattering will occur in the tissue. Specularly reflected photons maintain the incident polarization and, hence, are blocked by the second polarizer to give an orthogonal polarization with respect to the polarization of the incident light. Therefore, only the photons that have lost their initial polarization, that is the ones that suffer multiple scattering and are diffusely reflected at the surface, are detected by the CCD (image created by P.M. Lacerra (2012). Provided by Kurachi).

should probe mainly the superficial layers, while 600-nm (red) light should probe greater depths. Compared to NBI, conventional WL inspection cannot differentiate the vasculature in the more superficial layers from that in the deeper tissue layers. The contrast between the microcirculation patterns is increased using NBI when the wavelengths used match the hemoglobin absorption peak. On the other hand, OPS imaging was found to be helpful in examining leukoplakias. Detection of only the orthogonal component allowed the contribution of the whitish superficial layer to be partly removed; this otherwise would mask important morphological information from the layers beneath.

Reflectance imaging techniques such as OPS and NBI have been shown to enhance the evaluation of the microvasculature evaluation and discriminate between vessels at different depths, providing new important morphological information for lesion assessment.

Fluorescence imaging

Typical WL inspection or reflectance imaging techniques such as NBI and OPS can only give information about tissue reflectance/scattering and absorption features. Besides tissue morphology, an important aspect of the lesion fingerprint arises from its biochemical composition. Under ultraviolet (UV) light excitation, most tissue chemical components absorb light and re-emit this absorbed energy as an electromagnetic wave, that is they emit a photon. These molecules are referred to as fluorophores. As already mentioned, typical fluorophores in biological tissues are NADH and FAD in epithelial cells and elastin and collagen in the stroma. Each fluorophore has its own characteristic absorption and emission spectra, which influence the fluorescence signal exiting from a tissue. As is the case with reflectance imaging, fluorescence is dependent on the tissue's scattering and absorption properties when transmitting both excitation and fluorescence light. Therefore, fluorescence imaging can provide information on both the biochemical and morphological composition of the tissue.[11,12]

A schematic of a fluorescence imaging set-up is shown in Figure 39.5. Fluorescence imaging uses safe UV–blue light to illuminate the oral cavity. Light penetrates inside the tissue, exciting endogenous fluorophores present in the epithelium and stroma. Re-emitted fluorescence photons travel inside the bulk tissue and may exit the tissue surface. Only these latter photons can be detected by a charge-coupled device (CCD) camera placed near the patient. A longpass filter is placed in front of the camera to block scattered excitation photons. Without this filter, the image would be formed only by the scattered excitation photons, since this light–tissue interaction is much more intense

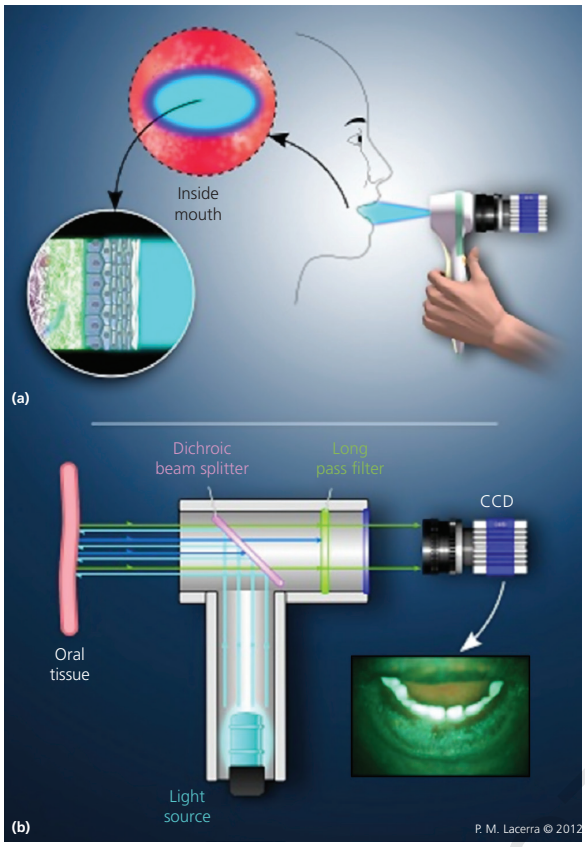


Figure 39.5 A fluorescence imaging system. (a) A handheld device coupled to a CCD is used for illumination and detection of native tissue fluorescence. (b) This device is composed of a dichroic beam splitter that reflects the excitation light and directs it to the tissue. Re-emitted tissue fluorescence is transmitted by the dichroic mirror and reaches the camera. A longpass filter is placed in front of the CCD for additional blocking of the excitation light (image created by P.M. Lacerra (2012). Provided by Kurachi).

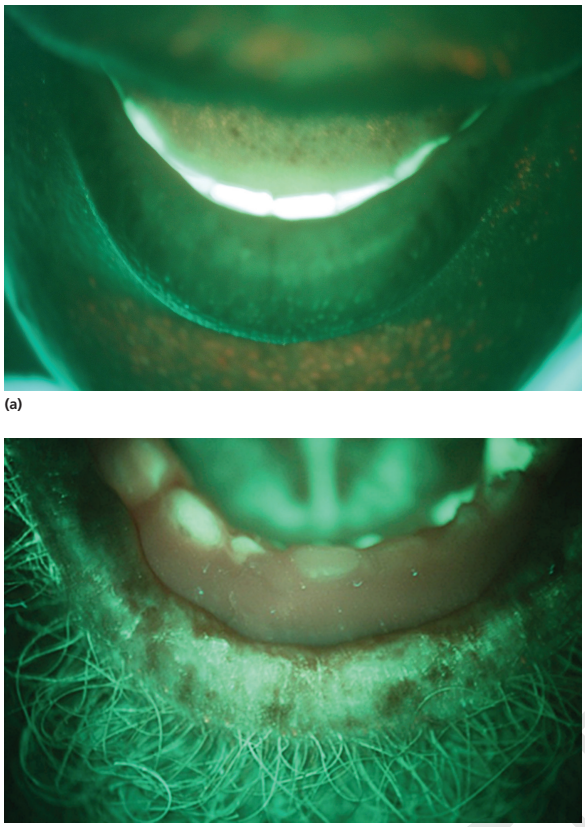
than the induced native tissue fluorescence. Red–green–blue (RGB) images are obtained by placing a Bayer filter mask on the detector.

The spectral information in the widefield image is reduced to three scalar values. This imaging modality provides two-dimensional (2D) biomorphological information: homogeneity, relative fluorescence intensity, distribution pattern for each of the RGB colors. Typical fields of view are of the order of several square centimeters,[9,10] making this modality more suitable for cancer screening than endomicroscopy or fluorescence spectroscopy, which usually provides information for just a few square millimeters.

From a clinical point of view, the widefield fluorescence pattern of the cancer and potentially malignant lesions appears as a dark-brownish area, while healthy tissue shows a pale green retained fluorescence.[10,13–17] Usually, a contralateral normal tissue, chosen by an expert clinician, is imaged to provide a reference standard. Different studies have attempted to explain the origin of native fluorescence in oral tissue. In two recent studies, Pavlova et al. related the lack of native fluorescence in neoplasia to a loosening of collagen cross-links as a result of cancer cell proliferation inside the connective tissue.[11,12] Typically, loss of native fluorescence in a potentially malignant lesion is accompanied by an increase in red fluorescence, which probably originates from porphyrins.[18]

An important issue in cancer diagnosis is the correct determination of the lesion border in order to avoid only partial removal and reduce cancer recurrence after surgery. For this reason, an extended margin of clinically healthy tissue needs to be defined by the surgeon. Compared to normal WL inspection, better visual contrast between healthy and potentially malignant tissue was achieved by means of fluorescence imaging.[9,14,16,17] Poh et al. observed a loss of fluorescence up to 25 mm away from the detected WL border.[16] Histopathology analysis confirmed the presence of a dysplastic lesion. The dysplasia usually was not isotropically distributed around the lesion, confirming that a safe margin of 10 mm does not ensure total lesion removal. To emphasize the importance of this finding, in a subsequent study Poh et al. screened the recurrence of dysplasia after surgical removal of oral lesions guided by fluorescence imaging. There were no recurrences, compared to a 25% recurrence rate in the control group for whom only WL guidance was used.[19] Fluorescence imaging was also shown to give a higher detection rate for potentially malignant lesion in a study by Paczona et al.[20] In the same study, fluorescence imaging was also shown to be able to reveal occult lesions not detected on normal WL inspection.

The promising results for the clinical assessment by means of fluorescence imaging lead to the development of commercial devices such as VELscope (LED Dental Inc., Burnaby, BC, Canada) and Identafi3000 (DentalEz, Malvern, PA, USA).[21–24] The US Food and Drug Administration (FDA) approved both devices for autofluorescence-based oral mucosal screening. The accessibility of fluorescence imaging devices to hospitals and clinics not necessarily involved in technology development may result in research in low prevalence populations. Indeed, it should be pointed out that most of the clinical studies on the efficacy of fluorescence imaging described above were limited to case reports. When studies are performed in populations with a low cancer prevalence, both specificity and sensitivity decrease. False-positive results are generally ascribed to inflammatory lesions in which epithelium thickening and blood from a trauma can attenuate stroma fluorescence.[14] On the other hand, intense hyperkeratosis produces a bright superficial fluorescence that can mask the presence of a potentially malignant lesion beneath, leading to a false-negative result.[20]



(a)

(b)

Figure 39.6 Fluorescence widefield images of (a) the lower lip of a healthy volunteer and (b) of a patient with actinic cheilitis. The normal lip shows a more uniform native fluorescence and the actinic cheilitis lip shows a non-homogenous pattern. It is possible to discriminate areas with high keratinization and areas with loss of fluorescence (b) (courtesy of Drs A. Takahama Jr and R. Azevedo).

From a microscopic point of view, the loss of fluorescence in inflammatory tissue is due to a decrease in fluorescence from epithelial cells and a thickening of the epithelium, which decreases the collection of the signal originating from elastin and collagen within the connective tissue. Cancer epithelial cells are more fluorescent, but there is still a loss of fluorescence due to the weakening of collagen cross-link.[11,12] Collagen is the main contributor to the native fluorescence of the oral mucosa, so when there is no or less emission from this biomolecule, the overall result is a decreased green fluorescence.

The use of digital cameras, and consequently the acquisition of digital data, allows the implementation of an algorithm that scores cancer and potentially malignant lesions.[9,17] The importance of developing computational algorithms relies on the possibility of assessing lesion independently of the level of operator training and experience. Roblyer et al. developed an algorithm in which the ratio of green to red intensity value was used to reveal diseased areas in the oral cavity.[17] The algorithm was based on increase in red fluorescence and loss of green fluorescence inside the lesion area. Using this algorithm, the authors were able to discriminate normal tissue from dysplasia and invasive cancer with 100% sensitivity and 91.4% specificity. These good results lead them to produce a merged image with mapped pixel values indicating greater than 50% risk of the presence of dysplasia/cancer superimposed on the normal WL image, in order to help the surgeon with lesion resection.

As discussed above, fluorescence imaging is a new promising tool in lesion diagnosis, enhancing both discrimination of lesion borders and detection of the initial lesion. Typical sensitivity and specificity are in the order of 90–100% in high cancer prevalence populations, shifting to lower values for low prevalence populations. At the moment, two different devices for fluorescence imaging have already been approved by the US FDA and commercialized. In the future, a lowering of the price of the individual components of the devices, such as the light emitting diode (LED) and CCD, could make this technique cheaper and more accessible.[25]

Figure 39.6 shows fluorescence images of the lower lip from a healthy volunteer and from a patient with actinic cheilitis. It is possible to observe that the healthy lip shows a higher homogeneity in the fluorescence intensity, and in actinic cheilitis it is possible to recognize areas with loss of fluorescence due to epithelium thickening as well areas of hyperkeratinization.

Optical coherence tomography

Biochemical changes inside the malignant lesion are strongly characterized by a peculiar morphology in which neoplastic cells infiltrate the stroma. This disrupts the typical structural layers of the oral mucosa, epithelium, and lamina propria. This feature becomes more evident with lesion progression. On the other hand, inflammation does not interfere with stromal integrity. As already discussed, fluorescence widefield imaging does not easily distinguish the loss of fluorescence caused by epithelium thickening due to inflammation or dysplasia from that caused by collagen cross-link disruption due to cancer cell infiltration.[11,12] Also, an intensely keratinized superficial layer can mask a dysplasia beneath.[20] Therefore, a technique that could resolve, with a precision of the order of tens of micrometers, the different layers of the oral mucosa could give a fingerprint for tissue pathology.[26]

For 20 years a tomography technique based on infrared light has been used as an auxiliary tool for oral lesion diagnosis. This technique uses the same principle as a Michelson interferometer for imaging the different layers inside the tissue. In this system, light is divided into two different paths by a beam splitter (BS). The two beams are back reflected by two mirrors, merge once again at the BS point, and are sent to a detector. If the difference in the pathlength (ΔL) of the two beams is a multiple of the

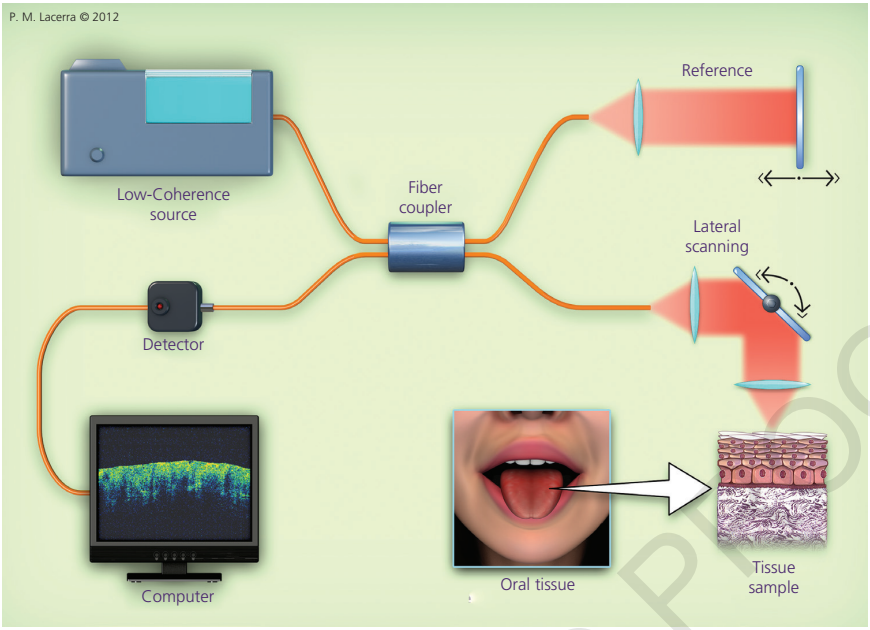


Figure 39.7 Schematic of an OCT system. Light from a low-coherence source is sent to a fiber coupler that splits it into two branches. Part of the light is sent to a reference scanning mirror, while the other part is delivered to the sample. A lateral scanning beam is used for lateral scanning. Light from the two branches is mixed once again in the fiber coupler and sent to the detector. The signal from the detector is acquired by the computer to provide a 2D reconstruction of the tissue (image created by P.M. Lacerra (2012). Provided by Kurachi).

photon wavelength (λ), the interference between the two beams will be constructive, leading to an increase in the signal, and vice versa.

In OCT, one mirror is replaced by the tissue, in which every layer is characterized by its own refractive index. The change of refractive index between layers acts as a mirror and partially reflects the light. The photon pathlength and reflectance are dependent on the wavelength. Hence, the interference pattern is dependent on the photon wavelength and the layer's refractive index and scattering properties. A typical OCT system is shown in Figure 39.7. A superluminescent diode in the near infrared range (830–1300 nm) with a bandwidth of 30–50 nm is used as a light source. By scanning the reference arm length and detecting the interference pattern, it is possible to reconstruct the structure and the width of each layer of the tissue. Technically, this scan is referred as an A-Scan, and it represents a vertical section through the tissue.

In order to produce an image of a 2D section of the tissue, a scanning mirror is placed at the tip of the proximal end, related to the light source. With this technique, usually referred to as a B-Scan, it is possible to reconstruct a complete transverse section of the tissue that resembles the vertical sections visible in histology. Compared to histology, OCT has almost the same axial resolution (5–10 μm) and has the advantage of being free of the distortion introduced by fixation.

Examples of OCT images are shown in Figure 39.8. It is possible to recognize the different layers of the tissue investigated: a keratinized epithelial layer, the epithelium, the basement membrane, and the submucosa. The keratinized epithelial layer and the lamina propria are characterized by a strong signal due to the high scattering from keratinized cells and collagen, while the epithelial layer is usually more transparent due to its low optical density. Dark areas could also indicate other structures such as salivary glands,[27] ducts,[28] and blood vessels. More generally, OCT resolution and definition is strongly dependent on the area of the tissue interrogated.[27]

Typical scan lengths and depths of commercial devices are in the order of 10 mm and 2 mm, respectively. B-Scan acquisition time is in the range of 1–20 seconds. New techniques, referred to as Fourier domain and involving sweep laser sources or a spectrometer as the detector, are providing faster imaging acquisition of up to 10 frames per second.[29] In comparison, techniques such as positron emission tomography, magnetic resonance imaging, and ultrasound show a lower resolution (0.5–1 mm) and a longer acquisition time.

When OCT is used for oral cancer diagnosis, the main features to take into account are grade of keranitization, epithelial thickening, epithelial irregular stratification, epithelial proliferation/down growth, and basal hyperplasia.[30] Since changes occur in oral cancer lesions with respect to normal tissue, an

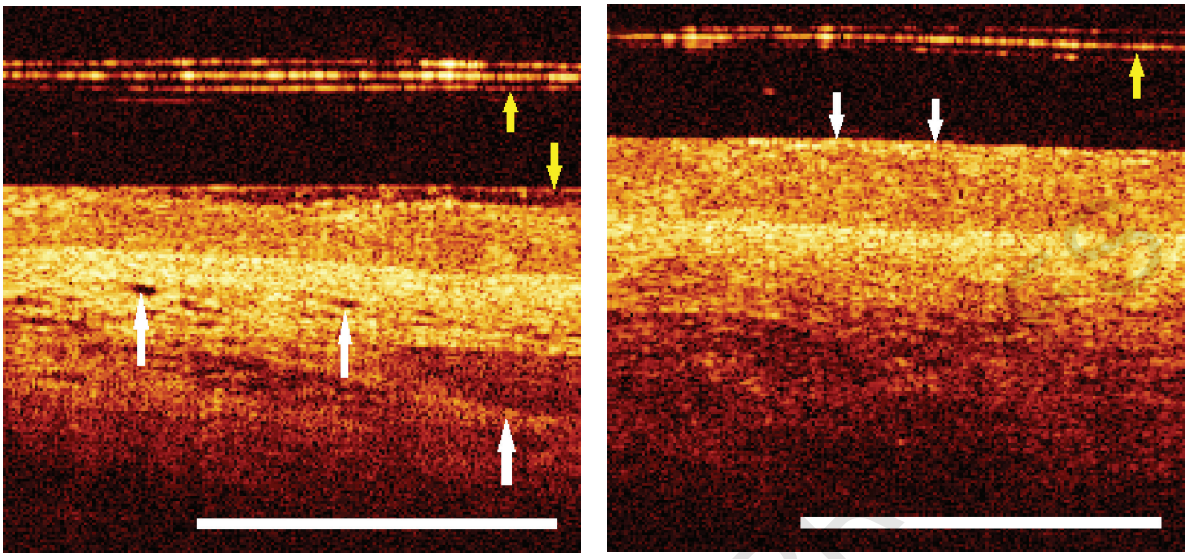


Figure 39.8 OCT images of (a) a healthy lower lip mucosa and (b) a left buccal mucosa. The epithelial layer and the lamina propria beneath are clearly distinguishable in the two images. The white arrows indicate salivary glands and blood vessels in (a) and the hyperkeratinized superficial layer in (b). The yellow arrows indicate artificial effects due to the protection cup of the fiber tip. Images acquired using Niris from Imalux (Cleveland, OH, USA). Scale bar 1 mm.

additional OCT image is taken from the healthy tissue close to the lesion for comparison.

In mild to severe dysplasia and CIS progression, the epithelial layer will still appear well defined and separated from the connective tissue beneath, but it is usually thicker than normal.[31] In a histological study of larynx biopsies, the epithelial thickening was found to be of a factor of 2 in moderate dysplasia and up to a factor of 3 in CIS,[32] compared to the typical epithelium thickness of different areas inside the oral cavity of healthy volunteer.[33]

In cases of early-stage and well-developed squamous cell carcinoma (SCC), the boundaries between the epithelial layer and the lamina propria and their architecture are disrupted. This peculiar feature is extremely useful in identifying lesion boarders between areas that are clearly stratified and those that are disorganized and in which layers are no longer clearly distinguishable. Another indicator of SCC is a highly heterogeneous appearance of the epithelial layer due to presence of cancer cell nests.[34] Malignancy is also highlighted by the presence of connective tissue papilla (CTP).[31] Early-stage SCC (ES-SCC) is characterized by a high density of CTP, which decreases with cancer progression.

Clinical assessment of oral cavity lesions by OCT has been successfully demonstrated. In a preliminary study involving 50 patients, Wilder-Smith et al. performed OCT immediately after clinical examination. Two blinded, specially trained clinicians diagnosed each lesion based on the OCT images and their features before histopathology analysis.[30] An agreement with the histopathology was observed for 89.6% of lesions, with a sensitivity and specificity in discriminate SCC versus non-malignant lesions of 93.1% and 93.1%, respectively. Kraft et al., in a prospective study of 193 patients with suspicious larynx lesions, compared the sensitivity and specificity of microlaringoscopy alone and combined with OCT, and found a higher sensitivity with the latter (66%) compared to the former (78%).[35] One of the main limitations of the OCT technique is the need for a trained clinician for a correct diagnosis. Indeed, assessment by OCT is still highly operator dependent.

To achieve a quantitative result and diagnosis by means of OCT images, Tsai et al. developed different imaging parameters[31,34] that could be related to tissue pathology. The evaluation of epithelial thickness could be used as an indicator of the presence of epithelial hyperplasia or moderate dysplasia. They also observed that a high standard deviation of the A-Scan along the epithelial layer highlights the heterogeneity of cell organization, giving a fingerprint for cancer cell nests. The presence of CTP and their density could be scored by looking at the standard deviation of pixel values along a line parallel to the surface at a depth of 350 μm. A high variability indicates high density, hence ES-SCC. On the other hand, a low variability suggests low CTP density and therefore a higher cancer stage. Tsai et al. also observed a higher axial light attenuation in WD-SCC, probably due to the presence of blood capillaries. For this reason, they suggested the exponential decay constant of the average A-scan intensity profile as a parameter to distinguish between well-differentiated (WD)-SCC and ES-SCC.[31]

By using an optical fiber for light delivery, it is possible to make small, compact, and flexible devices. First clinical trials with these have obtained high values for both specificity and sensitivity. One of the main limitations of OCT is its small field of view of just few millimeters, depending on where the clinician decides to place the probe for tissue interrogation. As a result, a cancer can still be misdiagnosed if the probe is not positioned at the right place, as in the punch biopsy procedure. OCT is suited to being coupled to the widefield imaging techniques, such as fluorescence imaging.

Oral tissue microendoscopy

All the imaging techniques described above generally give macroscopic biomorphological information about the tissue. With OCT it is possible to obtain a transverse section that visualizes the tissue layers and their thickness and integrity limits. Cell morphology and nuclear-to-cytoplasmic ratio are well-demonstrated features and provide a powerful fingerprint of tissue malignancy. The main problem with fluorescence widefield imaging is the false-positive results from inflammatory tissue conditions and epithelium thickness.

In the last decade new microscopic *in vivo* techniques for tissue imaging with subcellular resolution have been introduced.[36,37] These techniques use an optical fiber bundle to deliver the light to the tissue. Light can be delivered by a single fiber with a scanning system on the tip[38] or by a special coherent bundle.[37,39,40] In the latter case, each fiber at the proximal end preserves its position at the distal end of the bundle. Systems using a coherent bundle are simple, compact, and low cost.[37]

The optical set-up of a fluorescence microendoscopy system is similar to that used in fluorescence widefield imaging, but with a different fiber bundle and a magnifying objective. The UV–blue light from a source is reflected by a dichroic BS and sent to the fiber bundle using a microscope objective. Light passes through the fibers and reaches the sample. The fluorescence signal is then collected by the same bundle, enters the objective, and passes through the dichroic BS to reach the CDD. A further lens is introduced into the fluorescence optical path in order to give the optimal magnification for the CCD dimensions.[37] The field of view depends on the size of the CCD, which can be up to 1.4 mm. Resolution is in the range of 4–5 µm and depends on fiber size and spacing. With small optical modification, it is possible to transform a fluorescence microendoscope into a reflectance one.[40] The dichroic mirror should be replaced by a 50% BS and two cross-polarized mirrors should be placed in front of the light source and the CCD in a set-up similar to that for OPS.

Unfortunately, the autofluorescence signal from the cells is not strong enough to produce an image with a good signal-to-noise ratio. Therefore, an external fluorophore, acriflavine, should be topically applied. This dye shows affinity for cell nuclei and as a result, the nuclei appear as brilliant structures.

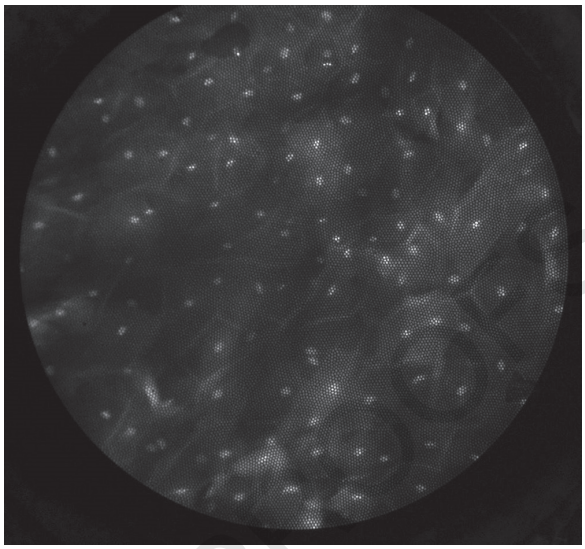


Figure 39.9 Image of the labial mucosa of the lower lip stained with proflavine, acquired using a microendoscope equipped with a coherent bundle, as described by Muldoon et al.[37] The cell nuclei and morphology are clearly distinguishable.

In vivo imaging by microendoscopy has been demonstrated.[37] An *in vivo* image of human labial mucosa, obtained using a microendoscope developed by Muldoon et al.,[37] is shown in Figure 39.9.

The first clinical study on *ex vivo* samples was performed using 150 unique sites from fresh biopsies of 13 patients with confirmed SCC.[39] Two different types of evaluation were used. The first evaluation was accomplished by three observers with long experience in head and neck pathology. Observers were trained by analysis of five microendoscope images for both normal and neoplastic tissues. Images that were out of focus or showed motion artifact were discarded. After training, each observer classified images according to a 5-point scale: 1 for "normal tissue" to 5 for "surely neoplastic." A receiver operator characteristic (ROC) curve was realized, with the threshold varying from 1 to 5. Values for the area under the curve ranged between 0.89 and 0.96.

The second method was based on computational algorithms. For each image, two different parameters were scored: the image entropy, defined as the randomness of pixel values, and the nuclear-to-cytoplasmic ratio. To the 2D scatter plot obtained, a five-fold cross-validation algorithm was applied. The values obtained for sensitivity and specificity were 81% and 77%, respectively. The advantage of using computational algorithms relies on the fact that the result is completely independent of clinician training and expertise.

Microendoscopy has been shown to be a highly promising tool for oral cancer diagnosis, but there are still some limitations. The fiber bundle has to be placed in direct contact with

the tissue; therefore, images can only be obtained for the superficial layer. This problem can be solved by optical sectioning or by mechanical bundle penetration by means of a 16-gauge hypodermic needle. Another problem is the small field of view, of just 1.4 mm in the best cases. This means that microendoscopy is not a suitable standalone technique, but must be coupled with a widefield technique. The combination of these techniques could be a promising protocol for oral cancer assessment in which fluorescence imaging could be used for oral screening, while microendoscopy could be used to inspect a suspicious area in order to rule out false-positive results.

Spectroscopy techniques for diagnosis of oral cavity malignancies

Optical spectroscopy refers to the study of the interaction between light and matter and has its origins in the observation of visible light dispersion by a prism. Nowadays the concept of spectroscopy comprises the measurement of radiation intensity as a function of wavelength (λ) or wavenumber (κ), and these quantities are related to each other by the equation:

$$\kappa = 2\pi/\lambda$$

Our knowledge about the structure of atoms and molecules mainly comes from investigations based on spectroscopy. Thus, spectroscopy has made a noticeable contribution to the present state of atomic and molecular physics, chemistry, and molecular biology. The basic devices used for spectroscopic studies are usually called spectrometers, spectrophotometers, or spectrographs.

The value of optical spectroscopy in detecting disease has been known for decades. Spectroscopy techniques can detect biochemical changes in tissue and depending on the technology used to perform tissue evaluation, this can be with high sensitivity and specificity.

The possibility of *in vivo* and real-time spectroscopy measurements relies on the fact that light can be delivered and collected by optical fibers, which can be placed in contact with a tissue surface or inserted into tissues using catheters, needles, and endoscopes.

The main optical spectroscopy techniques used to date for oral cancer diagnosis are fluorescence and Raman spectroscopy.

Fluorescence spectroscopy

Fluorescence spectroscopy is a powerful technique that may identify different molecules. Fluorescence is related to light absorption and results from an electronic transition from the excited state to the ground state of a molecule. Each molecule has a specific emission spectrum and any change in the molecule can, in principle, be detected by changes in the fluorescence spectrum, which depends on the excitation light that is used. Since biological tissues consist of a combination of several different biomolecules, the fluorescence spectrum of a tissue is a combination of the emission spectrum of each of these molecules.

In comparison to the imaging modalities, spectroscopy produces a spectral graph. The fluorescence spectrum is presented as a relative intensity for each emitted wavelength.

The shape of the autofluorescence spectrum of a tissue depends on the presence and quantity of fluorophore, but it is altered by absorption and scattering events. The presence of disease changes the concentration of the fluorophores as well as light scattering and absorption properties of the tissue, due to changes in blood oxygen concentration, nuclear size distribution, collagen content, and epithelial thickness.[41] The fluorescence of collagen and elastin, for example, can be used to distinguish tissues and their pathology, like epithelial and connective tissues.[1]

The possibility of using fluorescence spectroscopy as a technique for non-invasive, real-time, and early detection of oral cancer has been widely explored. In 1998, Gillenwater et al. reported the findings of a clinical study where fluorescence spectroscopy was used to distinguish oral lesions from normal tissue.[42] The excitation wavelength was varied between 337, 364, and 410 nm and the fluorescence emission was acquired between 350 and 700 nm. A decrease in the overall fluorescence spectra of neoplastic and dysplastic tissue was observed. Analysis using peak intensities, spectral line shape, and algorithms was performed and pointed to significant differences in the spectra from normal, dysplastic, and malignant mucosa, and also showed that a better discrimination between normal and abnormal tissue was obtained when using 337 nm and 410 nm as the excitation wavelengths. This study suggested that fluorescence spectroscopy has the potential to improve non-invasive diagnosis of oral cavity neoplasia.

Betz et al. also observed a decrease in the fluorescence intensity when comparing normal and malignant oral mucosa in patients.[43] They used both fluorescence imaging and spectroscopy and measured a reduction of the green autofluorescence at the lesion site in patients. The spectral analysis showed contrasting autofluorescence intensities between tumor and normal tissues in 94.4% of the patients.

Another interesting behavior of the fluorescence spectra of oral cancer was reported in 1999 by Inaguma and Hashimoto.[18] They evaluated 78 oral carcinomas in patients and observed porphyrin-like red fluorescence in 85% of them. The porphyrin-like fluorescent compounds were extracted from the carcinomas and evaluated, showing that, in some cases, a mixture of porphyrins is present in the lesions. The authors suggested the use of this red fluorescence as a criterion for oral cancer optical diagnosis.

Fluorescence can also be used in conjunction with other types of spectroscopy techniques, as described by Muller

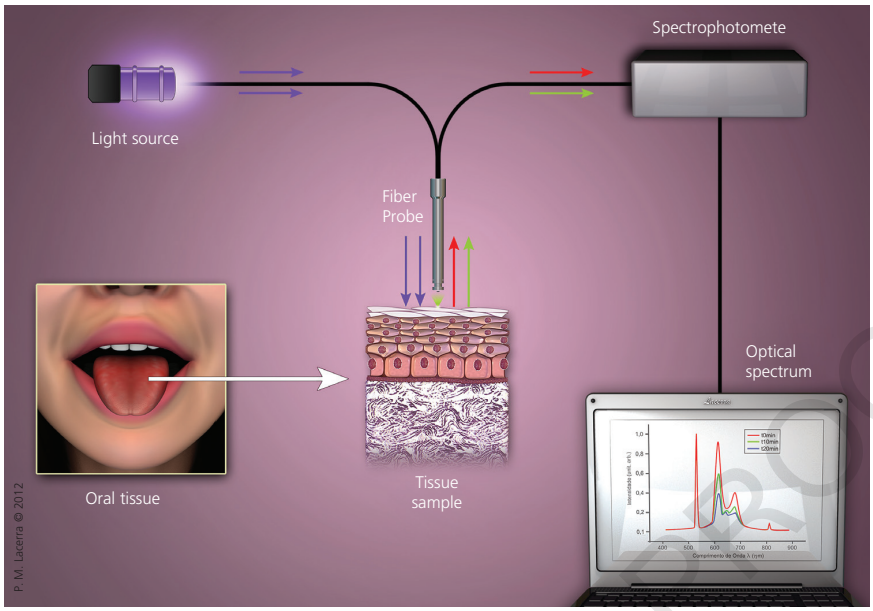


Figure 39.10 Schematic of the set-up of a fluorescence spectroscopy system (image created by P.M. Lacerra (2012). Provided by Kurachi).

et al.[44] These authors correlated biochemical and histological changes in the oral tissue with fluorescence, reflectance, and scattering spectra, introducing an approach called trimodal spectroscopy (TMS). Reflectance and fluorescence spectra were collected from 53 sites in 15 patients with known malignancies, and from 38 sites in eight healthy volunteers. In contrast to the findings of Inaguma and Hashimoto, Muller et al. observed red fluorescence not only in malignant lesions, but also in some oral cavity sites of healthy volunteers, which they suggested was due to bacteria that also produce protoporphyrin IX. With the TMS technique, the authors were able to distinguish cancerous/dysplastic from normal tissue with 96% sensitivity and specificity, and also to discriminate cancerous from dysplastic tissue with 64% sensitivity and 90% specificity.

De Veld et al. combined fluorescence with another optical technique, diffuse reflectance spectroscopy, and evaluated the performance of both spectroscopies in the diagnosis of oral cancer.[45] They evaluated the spectra of 172 lesions and 72 sites in healthy volunteers and compared the techniques individually and combined. When combined, the diffuse reflectance was used to correct the fluorescence spectra for blood absorption. The results showed similar results for raw and corrected autofluorescence, as well as for diffuse reflectance spectra. Their findings also implied that blood absorption and scattering effects are efficient at distinguishing cancerous from normal mucosa. A slight improvement was observed when the two spectroscopy techniques were combined; however, the authors concluded that although lesions of oral mucosa could be reliably distinguished from healthy mucosa, it was not possible to classify lesion type.

A depth-sensitive optical spectroscopy system was used by Schwarz et al. on oral lesions and normal volunteers, and differences were obtained when comparing autofluorescence and diffuse reflectance of neoplastic versus non-neoplastic sites, keratinized versus non-keratinized tissue, and shallow versus deeper oral tissue.[46] This type of system has four probe channels capable of collecting spectral responses from different tissue depths. One of the channels interrogates the epithelial layer, another both the epithelium and shallow stroma, and the other two primarily the stroma. A loss of fluorescence was once again observed for neoplastic tissue. For non-keratinized oral sites, the sensitivity and specificity were comparable to those achieved with clinical diagnosis by expert observers. This depth-sensitive optical spectroscopy system may be a useful tool in the oral screening examination when performed by a healthcare worker rather than an expert clinical observer.

More recently, Francisco described a study using fluorescence spectroscopy aimed at discriminating oral cavity neoplasia from normal oral tissue[47]; 150 subjects were evaluated, 30 subjects with potentially malignant lesions (leukoplakia and erythroplakia), 50 with neoplasia (confirmed by histology), and 65 normal volunteers. The fluorescence spectra were acquired using 405 nm and 532 nm as the excitation wavelengths. Figure 39.10 shows the optical set-up of the system used in this study, which represents the basic instrumentation used for fluorescence spectroscopy studies.

(a) (b)

Figure 39.11 (a) Fluorescence spectroscopy system and (b) a patient interrogation procedure.

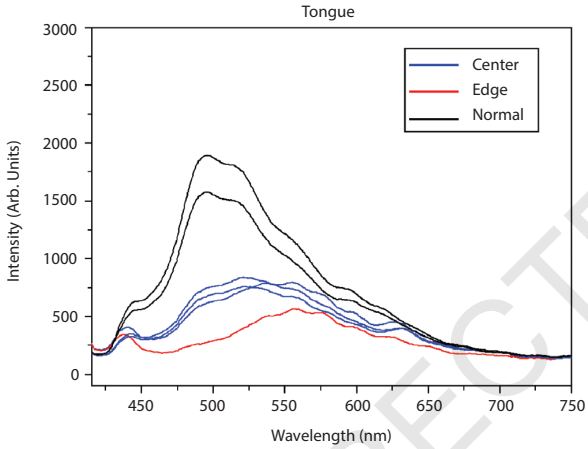


Figure 39.12 Fluorescence spectra of a patient with squamous cell carcinoma of the tongue. The black lines show the fluorescence spectra from the normal contralateral site; the blue lines show the fluorescence spectra from the center of the lesion; and the red lines show the fluorescence spectra from the lesion edges (clinically detected borders). Adapted from Francisco.[47]

This optical system is based on a fiber-optic probe that delivers the excitation light from a laser source to the tissue and also collects the fluorescent emission from the tissue, which is then detected by a spectrometer. Such a system allows fluorescence to be measured non-invasively. The probe tip is placed in direct contact with the tissue surface and the fluorescence spectrum is quickly acquired (Fig. 39.11). The graph shown in Figure 39.12 is an example of the spectral data collected from a patient with an SCC of the tongue. Each line represents the fluorescence spectrum at one interrogated tissue site. It is possible to observe that the shape of the fluorescence spectra differ for normal and malignant tissues. Principal component analysis (PCA) was used to discriminate normal tissue from oral cavity neoplasia. The results showed that fluorescence spectroscopy can discriminate normal tissue from carcinoma, but there is an important variance when considering the anatomical sites.

While fluorescence spectroscopy seems to be a powerful tool for the discrimination between cancerous and normal tissues, a large variation in autofluorescence spectra is often seen, complicating the diagnosis of lesions. Therefore, care needs to be taken with data analysis, because it has been shown that individual characteristics may affect the autofluorescence spectra of healthy oral mucosa. Factors like gender, alcohol consumption, tobacco consumption, wearing of dentures, and importantly skin color may affect the fluorescence emission by tissue.[48] Also, for oral cavity interrogation, the fluorescence spectrum will be affected if the patient has consumed drink or food containing a dye just before optical evaluation.

Raman spectroscopy

Raman spectroscopy is a vibrational spectroscopic technique, that is it uses light to excite vibrational energy states in molecules to get information about the molecular composition, structure, and their interactions in a sample.

Raman spectroscopy is based on Raman scattering, an event that happens when light interacts with a molecule and a small amount of energy is transferred from the photon to the molecule (or vice versa), leading to an excitation of the molecule from its lowest vibrational energy level to a higher one, and causing a shift (usually expressed in wavenumbers) between the incident and scattered photon.[1] The Raman spectra show the scattered light intensity as a function of the energy difference between the incident and scattered photons.

Raman spectroscopy has low sensitivity when compared with infrared spectroscopy or fluorescence spectroscopy because of the relatively low efficiency of Raman scattering. The Raman signals are many orders of magnitude lower that fluorescence

signals, which means the fluorescence background overwhelms the Raman bands, limiting the utility of Raman spectroscopy.[2]

Most investigations reported in the literature have used Raman spectroscopy in *ex vivo* specimens obtained by surgical resection. This technique has shown its potential to discriminate between normal, inflammatory, premalignant, and malignant sites in oral cavity tissues.[49] Such discrimination is possible due to differences in the observed Raman bands, which can be attributed to biological compounds such as proteins, lipids, and DNA, as well as to chemical bonds. Variations in the relative intensities, position, and width of the bands are related to the sample composition.[50,51] Complex analysis, like multivariate data analysis, is often used and required to extract the variations in the dataset.

Only a few studies have described the use of Raman spectroscopy *in vivo*. The main reason for this is the fact that the instrumentation for Raman spectroscopy is more complex, less portable, and of higher cost compared to the fluorescence instrumentation. Guze et al. were the first to evaluate the Raman spectra from the oral mucosa of patients to determine if Raman spectra varied for distinct oral mucosal types and to evaluate the clinical applicability or Raman spectroscopy.[52] *In vivo* measurements were taken from 51 human healthy subjects and no clear differences were observed between Asian and Caucasian subgroups. However, in the same ethnic group, significant differences were observed for spectra from different oral sites.

Another *in vivo* study by Bergholt et al. aimed to evaluate differences in normal tissue of oral cavity.[53] They used a system with a 785-nm diode laser as the excitation light source to interrogate the tissue and a fiber-optic Raman probe with a ball lens for maximizing the tissue excitation and collection of the signal. A total of 20 normal healthy subjects were recruited for this study. Data analysis showed that high quality *in vivo* Raman spectra could be acquired from the oral cavity in real time (in about 0.5 seconds) and also that different anatomical sites (hard palate, soft palate, buccal mucosa, inner lip, gingiva, floor of the mouth, and dorsal and ventral tongue) showed variable spectra, indicating the importance of considering the site of investigation in the interpretation of diagnostic algorithms for oral tissue diagnosis and characterization. These results suggest that Raman spectroscopy has the potential to be clinically applied for *in vivo* detection of diseases in the oral cavity, such as oral cancer.

Final considerations

Optical techniques using different light–tissue interactions provide relevant information for cancer diagnosis, concerning the biochemical and structural composition of oral mucosa. Widefield imaging exposes macroscopic surface features, enhancing the visual discrimination of malignant characteristics and lesion borders. Endomicroscopy provides subcellular resolution of morphology similar to histology, such as nuclear-to-cytoplasmic ratio. The information obtained with spectroscopy modalities gives data that may be related to specific fluorophores and absorbers (fluorescence spectroscopy) and to biomolecules such as DNA, proteins, and lipids, or even to specific chemical bonds (Raman spectroscopy).

All this tissue information is quickly obtained through non-invasive and non-destructive procedures. There is less clinician influence on clinical diagnosis when a more objective optical technique is used.

The additional information provided by optical techniques may result in improved resolution and sensitivity for oral cancer diagnosis, but understanding of the light–tissue interactions and the changes induced by cancer development and progression is essential to achieving this.

Optical techniques are relevant auxiliary tools for cancer detection, but must be combined with all the conventional diagnostic procedures.

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