Optical "biopsy" in laryngeal cancer detection

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INTRODUCTION

Squamous cell carcinomas of the larynx is by far the most frequent malignant tumor of the head and neck region, representing 1-2% of all malignancies diagnosed world-wide. Treatment results of early cancer are not unfavorable: for the reported 5-year-survival rate of Tis, T1, T2 laryngeal cancer patients ranges from 80 to 90% [1]. At present, indirect laryngoscopy is widely used for the evaluation of laryngeal cancer and precancerous lesion, while direct laryngoscopy under general anesthesia with biopsy remains to be the gold standard.

The expression optical "biopsy" has entered into usual speech among researchers in the domain of biomedical optics. In fact this joining of terms is a paradox, because "biopsy" refers exactly to the removal of tissue, while the signification of "optical" is that tissue is not removed. The real meaning of this expression consists in amount of optical measurements mediated by optical fibers which allow the physician to perform an instant tissue diagnosis in vivo, in situ, noninvasively and in real time, previously possible only by using histological analysis. The main reason of this is to avoid the surgical removal of biopsy tissue samples.

These refined optical techniques go beyond standard endoscopic methods by offering improved image resolution, contrast, tissue penetration and delivering biochemical and molecular knowledge about mucosal disorders. For some endoscopic application, for which "random" biopsies are repeatedly taken to discover premalignant or early malignant lesions, optical measurement could enable "guided biopsy", with increased probability for sampling a diseased site, reducing the number of tissue samples. Thus, additional purpose is certified by the potential for reduced health-care costs by eliminating unnecessary histology. Besides, the instant diagnostic information can reduce the emotional trauma to the patient awaiting an answer [2].

Recent technological advances in fiber optics, light sources and detectors have stimulated the tremendous development of many optical methods that promise to notably improve capability to visualize and evaluate human epithelium in vivo [3,4,5]. Some of these techniques are used for detection of laryngeal cancer: fluorescence spectroscopy, optical coherence tomography, narrow-band imaging, contact endoscopy and confocal endomicroscopy. This review describes the basic biophysics of light-tissue interactions, evaluates the strengths and weaknesses of each technique and explores clinical and preclinical evidence for each approach.

OVERVIEW

Fundamental parameters that characterize tissue

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optics in terms of radiative transfer are: absorption coefficient, scattering coefficient and anisotropy and measuring those constitutes a central issue in biomedical optics [6].

a. The absorption coefficient (μa) determines the probability of absorption events for a photon that propagates in tissue. Absorption occurs due to coupling of photons energy at a particular frequency with electronic or vibrational transitions energy. Depending on the spectral field, absorption is caused by:
- Cellular DNA and protein (amino acids and nucleic acids) in ultraviolet;
- Specific chromophores (hemoglobin, melanin, etc.) in visible;
- Water, OH and amine infrared.

b. Scattering coefficient (μs) determines the probability of a photon to be scattered during propagation. Scattering is caused by fluctuations of the refractive index of the environment in microscopic scale (ultra-structural heterogeneity) such as water-lipid interfaces membranes inside the cell and surrounding cells and fibers of collagen in the extracellular matrix. We can say that the absorption properties of tissue are tied to tissue biochemistry, while the scattering ones are related to its morphology.

c. Anisotropy (g) is the average cosine scattering angle and weighted the scattering coefficient effectiveness.

d. Alongside the three basic parameters, in medical practice, are also used: attenuation coefficients (total and effective); reflection; the refractive index; reflectance.

Cheong et al [7] realized (1990) a comprehensive compilation of optical tissue properties (absorption coefficients, scattering, total and effective mitigation and anisotropy for different biological tissues at a variety of wavelengths). They concluded that for a specific tissue, large areas of these parameters are frequent, indicating the sensitivity and vulnerability of such measurements to sample variation, detection equipment, boundary conditions and adopted light propagation model. Confidence in the obtained values can be compromised by any of these factors. After 1990, numerous studies have improved this knowledge, creating optical exploration methods that will be presented below.

1. Spectroscopy

1.1. Autofluorescence endoscopy (AFE)

Autofluorescence was first observed by Policard [8] in 1924, but Alfano [9] demonstrated in 1984 differences in spectral profiles of cancerous and normal tissues. In the last decades, this technique was used for screening in early detection of cancers of upper- and lower-GI tract and also lower-airway, and recently it was used in identifying precancerous and cancerous lesion [10].

Tissue autofluorescence is based on the presence of endogenous fluorophores, which emit visible green light on appropriate excitation. This involve structural proteins (collagen, elastin) mainly located in the submucosa, but also keratin, porphyrin and enzymes of the respiratory chain such as nicotinamide adenine dinucleotide and flavin adenine dinucleotide within the epithelium [11].

Figure 1: Squamous cell carcinoma of the right vocal fold: White light endoscopy (a); Autofluorescence endoscopy (b) [13]
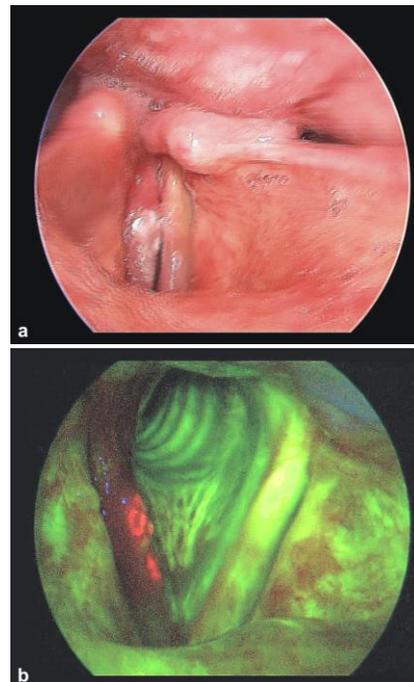
Because of an altered metabolism in tumor cells, premalignant and malignant tissues show a decreased quantity of fluorophores. Moreover, epithelial thickening prevents the excitatory blue light from reaching the submucosa. Both mechanism results in a loss of autofluorescence, with a color change from green to red-violet [12].

In AFE, healthy mucosa and benign lesion covered by normal epithelium (polyps, nodules, cysts, Reinke's edemas) present green color (no loss of autofluorescence). Scar tissue following surgery or radiotherapy shows a violet color; the same aspect is found in hyperplasia and chronic inflammation.

AFE can be used in early detection of premalignant and malignant lesions, in guided biopsy and oncological follow-up. This technique can only detect changes on epithelium, but not in the deeper structures [14].

Many studies have found that AFE has a better sensitivity and specificity in detecting laryngeal malignant changes than the classic white light endoscopy (WLE) [15,16,17]; a meta-analysis made by Kraft and co. in 2010 concluded: in identifying precancerous and cancerous lesions of the larynx, sensitivity (91% vs. 73%), specificity (84% vs. 79%) and accuracy (88% vs. 77%) of AFE were superior to WLE alone[18]; false-positive diagnoses result from chronic laryngitis, teleangiectatic polyps, granulomas[19] and papillomas; false-negative diagnoses are found in extreme hyperkeratosis, necrosis and inflammation [20,21].

There is a variant of this method involves the study of autofluorescence laryngeal mucosa after topical application of aminolevulinic acid, preferentially captured by the cancer cells, that became colored in orange-red (because protoporphyrin IX), while normal mucosa appear green [22].

Figure 2: Normalized mean and difference spectra for squamous cell carcinoma and non malignant tissue in larynx [26].
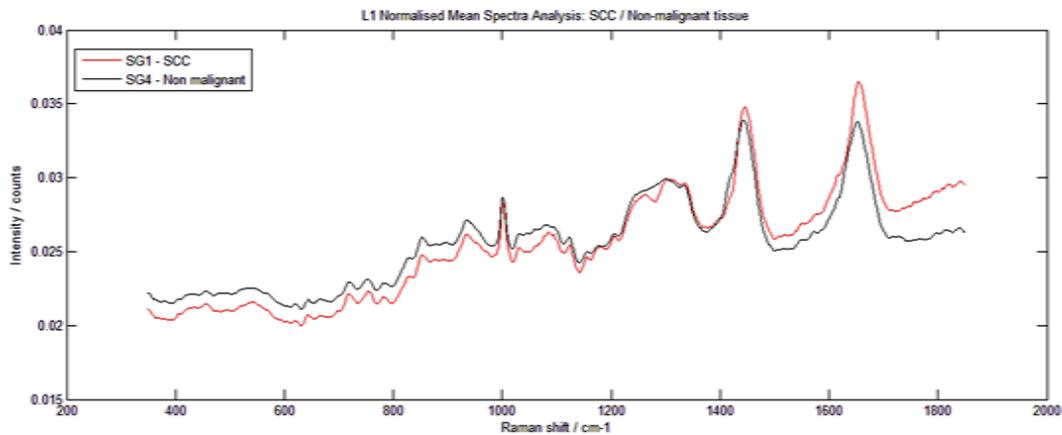
1.2. Raman spectroscopy

This method involves an effect whereby a small portion of tissue scattered light has a different frequency from that of the incident beam, with an equal amount of vibration frequencies of the molecules in the tissue; the effect is very weak, but with laser, it is possible to study the movements of rotation and vibration of molecules [23]. From the received signal, the percentage of biological molecules can be extracted in samples, such as collagen, elastin, beta-carotene, cholesterol (fig.2).

Introduced in medical practice in 2000, Raman spectroscopy is used to differentiate normal laryngeal tissue of precancerous and cancerous lesions [24]. Some authors publishing studies that indicate sensitivity between 70-90% and specificity in differentiating between normal tissues/malignant between 85-95 % [25].

### 2. Optical coherence tomography (OCT)

OCT is a modern method of investigation, in high resolution, mainly used in ophthalmology. It is

derived from low coherence optical interferometry and uses non-ionizing optical radiation in the near infrared field.

OCT allows measurements in real time, in situ, with resolution up to 10 times higher than conventional radiology (CT, MRI), making it possible to identify microscopic tissue structures (villi, glands, crypts, blood vessels). Despite this exceptional resolutions

(1-2 μm), the method is limited by the very low degree of depth (penetration of tissue) of 1-3 mm [27]. Articles published in the past decade shows that it can be used to detect an epithelial membrane rupture and overcoming of the laryngeal carcinoma or epithelial thickness measurement (normally between 98-185 μm, about 500 μm in hyperkeratotic lesions) [28].

Figure 3: T2 squamous cell carcinoma of the left vocal fold; (A) Endoscopic photograph. (B) Optical coherence tomography image demonstrating normal epithelium with an intact basement membrane on the right of the image, and invasive cancer with increased backscattering and loss of the basement membrane on the left. A transition zone is seen between these two areas. Note the area of motion artifact on the left of the image (bm-basement membrane; ca-invasive cancer; e-epithelium; tz-transition zone; SLP-superficial lamina propria) [29]
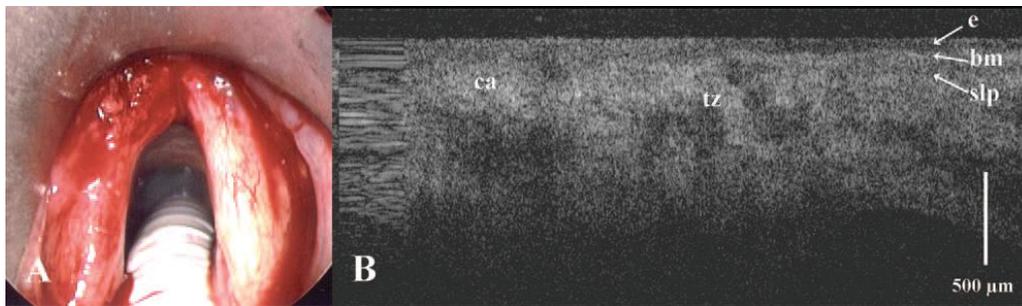
3. Narrow band imaging (NBI)

It is a modern endoscopic technique for investigating the vocal cord that uses certain wavelengths of light to improve the viewing of epithelial and sub epithelial microvascular pattern. They are using wavelengths of 415 nm (blue) and 540 nm (green), which are absorbed by hemoglobin, causing epithelial microvasculature to be colored in brown and become visible. The obtained images can be improved and recorded by using a HD (High-Definition) video system [30,31].

Neoplasia suspicion rises when on a brownish area, well-marked, is observed thick dark spots and/or tortuous course of the microvasculature, or more, a hypertrophic blood vessel that branches in small vascular loops [32,33]. There are studies that demonstrate that this optical method can even make the difference between neo-vessels specific to carcinoma and the ones altered by post-radiation inflammation [34].

The method was originally used for early detection and monitoring post-surgery esophageal [36], and

tracheobronchial [37] neoplasm lesions, but recent studies show that is gradually earning supporters using it for the same goals at the larynx [38,39] (good results in subglottis injuries). Thus, Japanese authors demonstrate that narrow-band imaging has a sensitivity of 91.3% and a specificity of 91.6% [40], in laryngeal cancer lesions, while a team from Italy (Brescia) reports 90% specificity and 98% sensitivity [41].

Another recent study [42], multicenter, prospective, randomized, which included patients with esophageal cancer and pharyngeal and laryngeal cancer, showed that narrow-band imaging compared with conventional endoscopy detected a higher number of superficial carcinomas synchronous in both locations, making them the authors to conclude that this method could be the standard examination for early detection of superficial cancer in those localizations.

But the interpretation of the images must be made by an ENT specialist with a good experience, to avoid false negative or false positive. Also false negative

may occur on lesions where the keratin layer has a greater thickness.

Figure 4: A. Conventional white-light image of the larynx. The left subglottic region is displayed slightly reddish. B. NBI image of the same site as a. Demarcated brownish area is seen in the left subglottic region surrounded by green area. C. Close white-light view of a. D. Close NBI view of b. There are scattered brown spots in the lesion. E. Histopathologic evaluation of the biopsy specimen revealed moderately differentiated squamous cell carcinoma of the larynx [35].
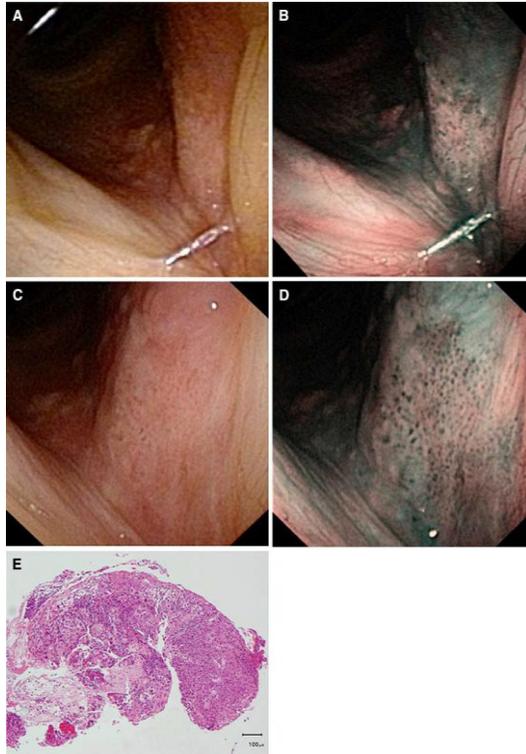

4. Confocal endomicroscopy

This technique is introduced into medical practice in 2004 by R. Kiesslich for in vivo diagnosis of colorectal cancer[43]. The principle of the method consists in illuminating the examined tissue with a laser wave of 488 nm which allows viewing epithelium in depth up to 250 μm. For a better image quality is given intravenously a contrast substance (fluorescein). The examination is made simultaneously with both endoscopic and endomicroscopic, acquiring images to identify the characteristics of epithelial cells (size, shape, appearance of the nucleus) near the basal membrane [44].

The limitation of this method is shallow penetration (250 μm), knowing that the cancer epithelial thickness can reach 500-1000 μm, being unable to view the membrane. Also it requires a pathologist with experience.

Introduced recently in Otolaryngology, there are still no studies on a large number of cases, but those in the gastrointestinal field [45,46] give high hopes for the future of this method.

There is a pilot study [47] clearly demonstrates the possibility to detect dysplastic cells near to the basal cell layer and within the subepithelial space in lesions with small leukoplakia (thin keratin layer). These results can have an impact on microlaryngoscopy to improve the precision for biopsy and on microlaryngoscopic laser surgery of the larynx to identify the margins of the pre-malignant lesion.

Figure 5: Confocal endomicroscopy image of the human supraglottis captured in vivo following topical application of 0.05% acriflavine hydrochloride [48]
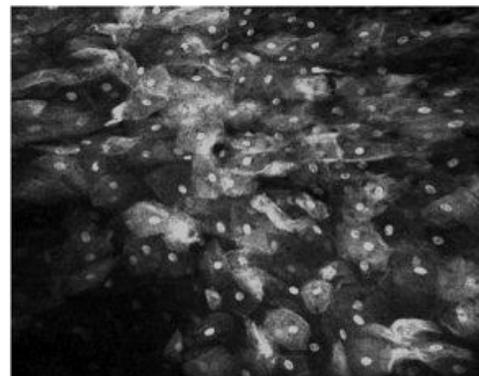
5. Contact endoscopy

Contact endoscopy is another novel noninvasive optical diagnostic imaging method that allows in vivo and in situ examination of the cellular architecture of the superficial layers of the mucosal epithelium. Magnified images are obtained using Hopkins' rod-lens endoscope placed on the surface of the dye stained mucosal tissue. This method allows evaluation of precancerous and cancerous lesions in vivo and has significant potential in the histopathologic diagnosis of many suspicious head and neck mucosal lesions without tissue biopsy. The

first reported use of CE in otolaryngology head and neck surgery was by Andrea et al. as a diagnostic tool in the evaluation of various pathologies in the larynx in the 1990s [49]. They were able to visualize and diagnose laryngeal mucosal pathology from the magnification of vocal fold epithelium and microvasculature during microlaryngoscopy after staining the vocal cords with methylene blue dye.

Few members of our group [50] have systematically drawing vascular changes of the vocal fold, with clinical and pharmacological applications. They demonstrated the changes of microvascularity, occurring since stage of hypertrophic chronic laryngitis (hypervascularity with dilated vessels, but parallel to the free edge of the vocal fold) till the carcinomatous lessions (with hypervascularity and areas of keratin, changes of the archaeological and cytological parameters: **U**niformity of the cellular-field, **R**atio (nucleus/cytoplasm), **S**ize and **S**hape of the epithelial cells [51].

There are more advantages of contact endoscopy. Most significantly, it offers a noninvasive, quick, and repeatable in vivo evaluation of the cytological architecture while avoiding the need for an invasive biopsy and its associated risks. CE provides immediate results, with the possibility of examining many mucosal areas in a short time. CE can also assess a wider surface mucosal area, providing more information than a selected histological section taken by biopsy [52]. It also avoids tissue damage and alteration of cellular architecture which may occur in the biopsy and histological preparation. Subsequently, this results in a dramatic improvement of the diagnostic yield of the biopsy. Other potential roles of CE include the rapid diagnosis of benign and malignant mucosal lesions in an outpatient or operating room setting, surveillance, guided biopsies, and intraoperative evaluation of tumor resection margins. Despite its advantages, CE does have its limitations. Most notably, CE can only evaluate the most superficial cell layer of the mucosal epithelium. This is most likely due to a number of factors including (i) poor penetration of methylene blue which only stains a few superficial layers, (ii) short focal distance of the scope (i.e., CE can only assess to a depth of 80 um at 60x magnification and 30 um at 150x magnification), and (iii) optical artifact at high magnification due to glare from light reflected from cells not in focus. The lack of depth of penetration prevents the evaluation of important histological information especially when vertical extent of dysplasia is crucial in distinguishing the different grades of dysplasia from carcinoma in situ and invasive carcinoma. As a result, these factors could affect the sensitivity of CE, thus accounting for some of the false negative diagnostic results noted by authors. The potential impact of CE missing a malignant lesion needs to be taken into consideration if this technology is to one day substitute histopathology. Future investigation into better penetrating dyes, advances in digital optics, and image enhancements will eventually allow better vertical staining and increased resolution of the deeper cell layers which would translate CE in becoming a much more sensitive and accurate diagnostic tool.

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