# Has Fluorescence Spectroscopy Come of Age? A Case Series of Oral Precancers and Cancers Using White Light, Fluorescent Light at 405 nm, and Reflected Light at 545 nm Using the Trimira Identafi 3000

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## ABSTRACT

**Background:** Optical spectroscopy devices are being developed and tested for the screening and diagnosis of cancer and precancer in multiple organ sites. The studies reported here used a prototype of a device that uses white light, green–amber light at 545 nm, and violet light at 405 nm. Given that oral neoplasia is rare, the need for a device that increases the sensitivity of comprehensive white light oral screening is evident. Such a device, in the hands of dentists, family practitioners, otorhinolaryngologists, general surgeons, obstetrician gynecologists, and internists, could greatly increase the number of patients who have lesions detected in the precancerous phase.

**Objectives:** The objective of this study was to present a case series of oral precancers and cancers that have been photographed during larger ongoing clinical trials.

**Methods:** Over 300 patients were measured at 2 clinical sites that are comprehensive cancer centers and a faculty practice associated with a major dental school. Each site is conducting independent research on the sensitivity and specificity of several optical technologies for the diagnosis of oral neoplasia. The cases presented in this case series were taken from the larger database of images from the clinical trials using the aforementioned device. Optical spectroscopy was performed and biopsies obtained from all sites measured, representing abnormal and normal areas on comprehensive white light examination and after use of the fluorescence and reflectance spectroscopy device. The gold standard of test accuracy was the histologic report of biopsies read by the study histopathologists at each of the 3 study sites.

**Results:** Comprehensive white light examination showed some lesions; however, the addition of a fluorescence image and a selected reflectance wavelength was helpful in identifying other characteristics of the lesions. The addition of the violet light-induced fluorescence excited at 405 nm provided an additional view of both the stromal neovasculature of the lesions and the stromal changes associated with lesion growth that were biologically indicative of stromal breakdown. The addition of 545 nm green–amber light reflectance increased the view of the keratinized image and allowed the abnormal surface vasculature to be more prominent.

**Conclusions:** Optical spectroscopy is a promising technology for the diagnosis of oral neoplasia. The conclusion of several ongoing clinical trials and an eventual randomized Phase III clinical trial will provide definitive findings that sensitivity is or is not increased over comprehensive white light examination. (*Gend Med.* 2012;9:S25–S35) © 2012 Elsevier HS Journals, Inc. All rights reserved.

**Key words:** diagnosis, fluorescence spectroscopy, oral cancer diagnosis, oral intraepithelial neoplasia, oral neoplasia, optical spectroscopy, reflectance spectroscopy, screening, sensitivity, specificity.

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## INTRODUCTION

In the last 20 years, advances in fiber optic and semiconductor technology have enabled a new generation of inexpensive, miniature optical sources and sensors to be developed. These devices have found a wide variety of uses probing the interaction of light with tissue. Studies in several organ sites have demonstrated that precancer and cancer can be detected in the epithelia of the colon,[1] esophagus,[2] bladder,[3] bronchus,[4] and cervix using fluorescence and reflectance spectroscopy.[5–20]

Although there are many known fluorophores, fluorescence spectroscopy is based on measuring changes in the metabolic activity and some structural aspects of the tissue. Specifically, reduced nicotine adenine dinucleotide and flavin adenine dinucleotide are important fluorophores that have long been known to be associated with increased cell turnover as measured by the well-established redox ratio. Additionally, biologic tissue samples studied under fluorescence microscopes have shown that changes in the stroma are dectectable as lesions progress in the neoplastic process. Neovasculature occurs in the stroma, and thus, hemoglobin changes can be seen with fluorescent light. Both collagen and elastin are important fluorophores found in the stroma of nearly all epithelia, and these change as dyplasia progresses.[21–23] Similarly, both comprehensive white light examination and reflectance spectroscopy correlate well with precancerous and cancerous changes in tissue morphology, probably based on the reflectance of light from the chromatin of the abnormal cells as they progress to neoplasia. Aneuploidy, the measure of abnormal amounts of DNA in the cell, has become a well-established biomarker of cancer progression and survival.[24–30]

Oral neoplasia is rare, unless one studies populations exposed to alcohol, tobacco, or other risk factors. Because most oral clinical care is provided by dentists, family practitioners, otorhinolaryngologists, general surgeons, obstetrician gynecologists, and interns, devices that increase the sensitivity of detection are needed. The rarer the cancer, the harder it is to detect; this is a well-known result of Bayes' theorem that takes the prevalence of disease into account when calculating the positive and negative predictive values. These positive and negative predictive values are often more important clinically than the sensitivity and specificity, because they specify how likely or unlikely it is to diagnose the disease when the test is performed.[31]

This case series was small but showed the possible potential of optical spectroscopy to increase the detection of oral precancers and cancers. Larger definitive trials are ongoing, and their results will be needed to quantify the sensitivity, specificity, positive and negative predictive values, and cost-effectiveness of these technologies. Additionally, Phase III trials that are carefully designed and adequately powered will be needed to quantify the increased value of this optical technology to comprehensive white light examination.

## MATERIALS AND METHODS

### Overview of Study Procedures

Men and women who were ≥18 years of age were eligible to participate in the studies, which began in 2008 and are ongoing. Patients were recruited from the oral cancer clinics of the The University of Texas M.D. Anderson Cancer Center and the British Columbia Cancer Research Centre and Agency, as well as the faculty practice of the University of Texas Health Science Center Dental Branch in Houston. A research nurse described the study to eligible patients and written informed consent was obtained from those agreeing to participate. A biomedical engineer assisted with trial conduct, including assisting the provider with taking the photographic images included in the trial.

Participants received: (1) a complete history and an oral cancer risk factors questionnaire, (2) a comprehensive head and neck examination, (3) a comprehensive oral white light examination, and (4) an examination using the device that has white light, violet light-excited fluorescence (405 nm) and green–amber reflected light (445 nm). Measurements were obtained from the large field views, and then 1 or 2 normal oral sites and areas from the suspicious neoplasm were biopsied and submitted for permanent section. All tissue findings were reviewed by histopathologists who were blinded to the spectroscopic images.

The prototypic device under study has a white light source, a light source that excites fluores-

**Figure 1.** Identafi device and viewing goggles (Trimira LLC, Houston, Texas).

cence at 405 nm, and a green–amber source at 445 nm. These sources are housed in a probe-like device that resembles a dental mirror and that can be easily inserted into the mouth for oral examination. **Figure 1** shows a prototype of the device like the one used in the oral clinics at all 3 institutions. Because fluorescent light is used, goggles are provided to the provider performing the examination to block the violet excitation light delivered. The rose-colored provider goggles transmit both the white and green–amber light, and can be kept on during the entire procedure. A lens with the exact characteristics of the provider goggles is attached to the lens of the camera used for documentation. Separate goggles are provided for the patient.

### Study Population

The study protocol was reviewed and approved by the Institutional Review Boards at The University of Texas M.D. Anderson Cancer Center, the University of Texas Health Science Center Dental Branch, and The British Columbia Cancer Research Centre. The studies were carried out at the 3 clinical sites, including The University of Texas M.D. Anderson Cancer Center in Houston, Texas, the faculty practice at the University of Texas Health Science Center Dental Branch, and The British Columbia Cancer Agency, Vancouver, British Columbia, Canada. At The University of Texas M.D. Anderson Cancer Center, patients were self-referred or referred by private physicians. At the faculty practices of the University of Texas Health Science Center Dental Branch, patients were referred from within the practice, which serves both as a primary care center and as a site for first referrals. At the British Columbia Cancer Agency, patients were referred from the network of primary care physicians in the province of British Columbia.

### Laboratory Tests

All routinely stained pathology specimens were submitted for diagnosis to experienced pathologists at each cancer center who specialize in oral neoplasia and who were blinded to the results of the spectroscopy and associated images. All centers used the World Health Organization classification for the diagnosis of oral precancers and cancers.

### Spectroscopic Measurements

Research grade, semiconductor-based spectrometers were designed and tested for similar technical efficacy before the commencement of the larger trials. Briefly, a multispectral semiconductor source was used to shine light in the mouth showing the entire field of view. A mirror was attached to the prototype to help visualize areas behind the teeth and in the pharynx. Each measurement took approximately an additional $\leq$3 minutes; however, documentation with photography added approximately an additional 2 minutes. The total light exposure was less than the American National Standards Institute standard.[25] All images were reviewed 4 times, beginning with 4 independent investigators blinded to the histology. Images were excluded for several indications: if the image was out of focus, if blood obscured the site, if there was too much glare in the image, or if the prototypic device failed during a measurement. Images were discarded if any of the 4 reviewers felt there was one of the aforementioned abnormalities. Overall, there was excellent agreement by the reviewers, but the larger experience will be reported when the trials are completed.

## RESULTS

Early results suggested that the addition of the fluorescence examination was helpful in identifying characteristics indicative of precancer and cancer

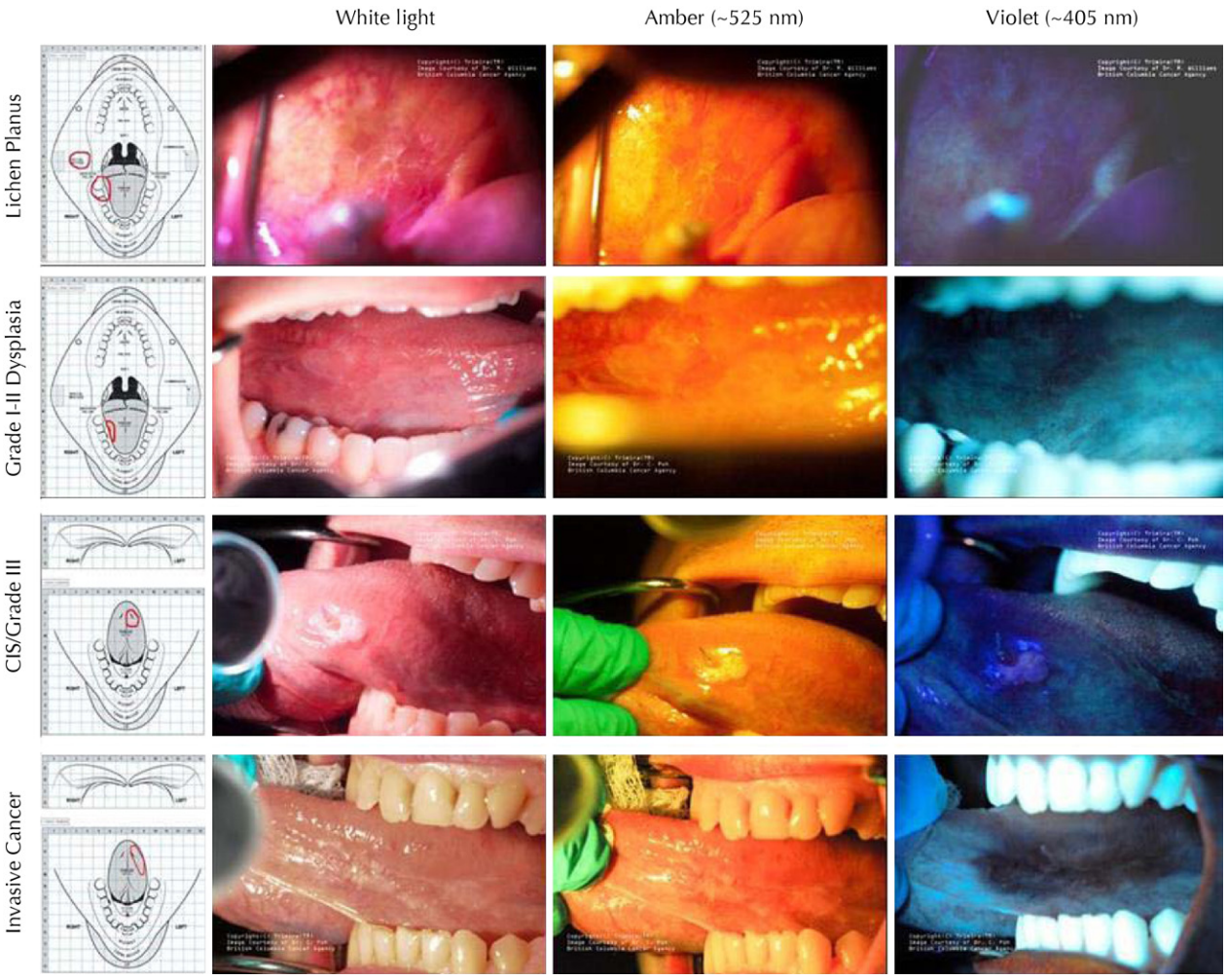


**Figure 2.** Lesions in the tongue region, as seen under light at 3 excitation wavelengths. CIS = carcinoma in situ.

over that seen at comprehensive white light examination. For example, at 545 nm, the green–amber light enhanced the keratinization of the lesions, often making them larger and more visible to the naked eye. Another advantage of using this wavelength was that the abnormal surface vasculature was enhanced. This allowed the clinician to detect vasculature not found in healing or inflammation, but rather vasculature that was quite specific to neoplasia, in which vessels become larger, smaller, and larger again. This was very different than normal vascular growth, in which vessels routinely decrease in diameter as they grow. In contrast, images of the violet-induced autofluorescence at 405 nm showed the deeper neovascularization of the stroma, and stromal changes in collagen and elastin breakdown that accompany the progression to neoplasia. This was easy to observe, because the vessels observed were different from those seen with the 545 nm light. Additionally, many biologic studies of tissue slices demonstrated that there was loss of autofluorescence consistent with breakdown of collagen matrixes in the connective tissues as lesions progressed.[36] Elastin breakdown was less well studied, but also strongly suspected in the carcinogenic stromal paradigm. Studies in the lung, using fluorescence confocal images comparing smokers with nonsmokers showed provocative evidence that elastin was also involved in the stromal biology.[32,33]

**Figures 2** through **5** show oral lesions from each of several areas in the oral cavity. The demographic characteristic information concerning the

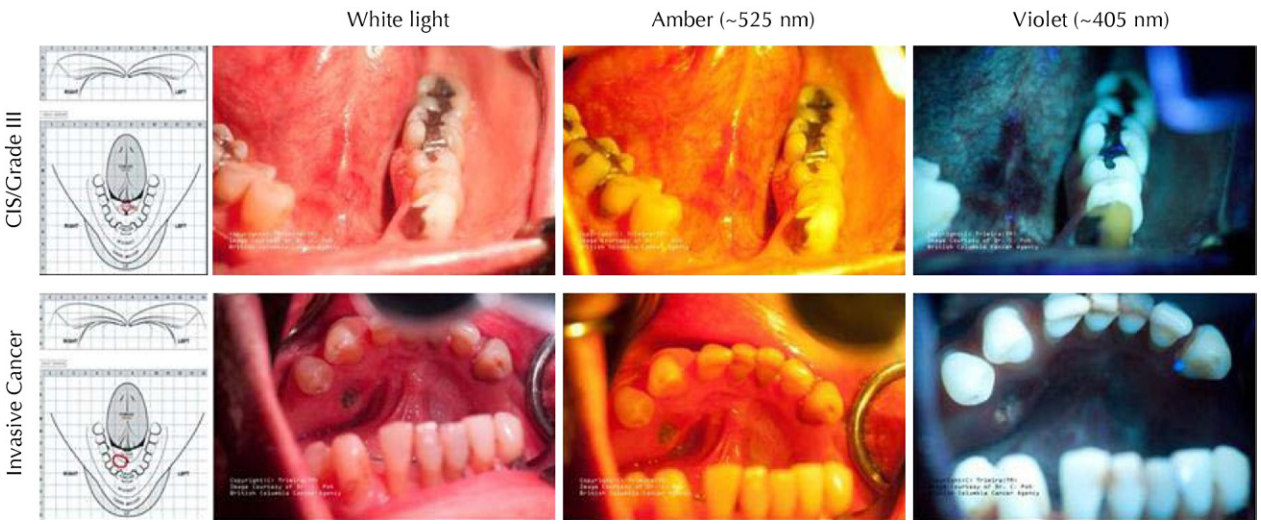


**Figure 3.** Lesions in the mouth floor region. CIS = carcinoma in situ.

patients was collected according to Health Insurance Portability and Accountability Act guidelines and is not available with the figures. These data will be reported in the future as the larger trials are completed.

**Figure 1** shows the prototype device that uses white light, fluorescence induced at 405 nm, and reflected light at 545 nm, and the goggles required during use of the device. for both the patient (yellow lenses) and the provider (rose lenses). **Figure 2**

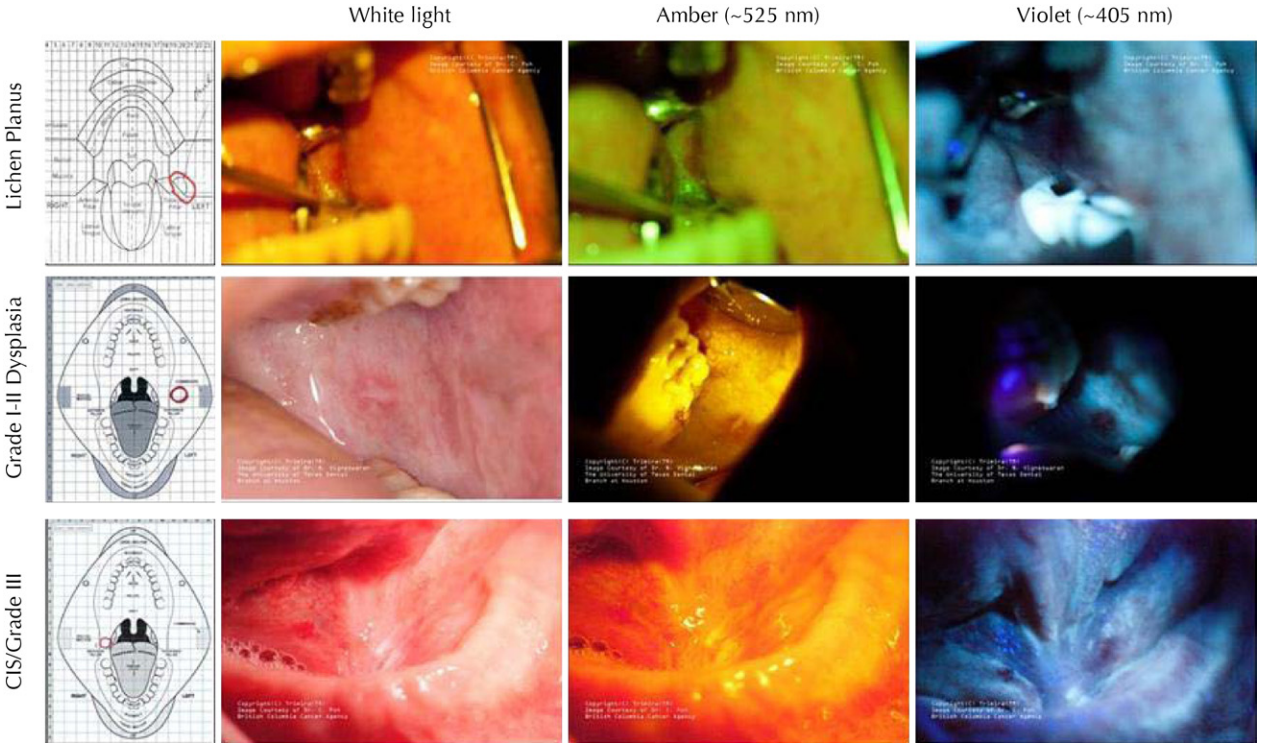


**Figure 4.** Lesions in the buccal mucosa. CIS = carcinoma in situ.


**Figure 5.** Lesions in the palate region of the mouth. CIS = carcinoma in situ.

shows 4 series of tongue lesions, including biopsy proven lichen planus, Grade 1 to 2 dysplasia, carcinoma in situ, and invasive cancer. Notice that the green–amber light at 545 nm enhanced visualization of the keratinized lesions over that of the white light images; additionally, superficial vasculature is more prominent. The violet light, at 405 nm, shows the increased neovascularization of the stroma as well as other changes believed related to stromal breakdown. This figure is especially demonstrative of the progressive nature of the changes. What is particularly notable is the increased size of the stromal vessels and breakdown, both of which were not unexpected, but were probably larger than expected. These findings might one day translate into help determining margins for surgical resection. These findings might also allow clinicians to better decide whether to operate or use a combination of chemotherapy and surgery or chemotherapy and radiation therapy rather than operate.

**Figure 3** shows lesions in the front of the mouth. This lesion has many of the cardinal features of an advanced neoplasm under white light examination, especially the feature of having outgrown its blood supply and appearing necrotic. The green–amber light at 545 nm shows the abnormal superficial vasculature, and the violet light at 405 nm shows the large area surrounding the lesion that is fed by new blood vessels and that is involved in stromal breakdown. The buccal mucosal lesion in **Figure 4** is under the retractor and is poorly seen by white light. The green–amber light at 545 nm emphasizes the keratin and thus shows the lesion in its entirety better than does the white light image. Additionally, the violet light at 405 nm shows a similarly sized area involved in the stroma by both neovascularization and stromal breakdown. This patient has lichen planus. The palatal cancer seen in the **Figure 5** is nicely visible by white light; however, the green–amber light at 545 nm shows the superficial neovasculature not seen well by white light, and the violet light at 405 nm shows that the neovasculature in the stroma involves a larger area than the lesion. The red–pink images are those of superficial or stromal infection by bacteria. Again, the stromal images may one day be helpful with margin detection in the operating room and perioperative treatment decisions.

## DISCUSSION

In 2004 and 2007, the World Health Organization reported 59 million deaths, 13 million attributed to cardiovascular disease and 8 million due to cancer. Both diseases are chronic diseases of 20- to 30-year duration. Thus, patients and providers have many opportunities to identify risk behaviors, detect lesions earlier in their course, and lessen the morbidity and mortality from these chronic diseases. Although the most common can-

cer-related causes of death worldwide are tracheal, bronchial, and lung cancer, Globcan 2002 reported 175,916 new cases of oral cancer in men and 98,373 in women, with corresponding deaths of 80,736 in men and 46,723 in women. In the United States, the Amercian Cancer Society reports that most common incident cancers are prostate, lung, and colon in men, and breast, lung, and colon in women. Death rates show that lung cancer kills more men and women than prostate, breast, and colon cancer.[34]

Oral cavity cancers in the United States are rare, accounting for <3% of cancer in men and <1% of cancers in women. The median age of incidence is 62 years and that of death 68 years. The age-adjusted incidence is 10.4/100,000 in men and women, the age-adjusted mortality is 2.6/100,000, and the lifetime risk of developing oral cancer before death is 1.02%. The percentage of diagnoses show that 34% are diagnosed when the cancer is localized, 46% when the cancer is in regional oral areas, 14% when regional lymph nodes are involved and/or there is extensive involvement of the mouth, and 7% present unstaged. The 5-year survival rates are 83% when the cancer is localized, 54% when the cancer involves regional oral areas, 32% when regional lymph nodes and/or extensive involvement of the mouth occur, and 54% when the cancer presents unstaged.[35]

Despite the fact that providers often focus on sensitivity and specificity, we often think more along the lines of positive and negative predictive values. For example, sensitivity tells us how often the test is positive in the face of disease, whereas the positive predictive value tells us how often when the disease is present, the test is positive. In tandem, specificity tells us how often the test is negative in the absence of disease, whereas the negative predictive value tells us how often when the disease is absent, the test is negative. Although sensitivity and specificity are fractions, the positive and negative predictive values are calculated using Bayes' theorem, and the equation takes into account the prevalence of disease. The rarer the cancer, the lower the sensitivity of any test. For rarer cancers, devices that can increase the sensitivity will also increase the negative predictive value, thus assuring us that when the tests are negative, the precancer or cancer is absent. This is most important to our clinical practice.[31]

The oral cavity has a very large surface area, many different tissue types, and many areas that are difficult to access visually. Such is the case of the lingual tonsils, the tonsilar pillars, and the oropharynx. Another obstacle to oral cancer diagnosis is the fact that the oral cavity is well vascularized, and that diagnosis is made with punch biopsy devices. There is ample bleeding after biopsy, and this encourages primary care providers to refer biopsying to surgeons. Any added step in the diagnostic process requires more resources and a higher likelihood of loss to follow up. This is particularly unfortunate when early diagnosis of oral cancer allows for greatly increased options for treatment and greatly decreased morbidity and mortality.

Conventional white light examination misses some lesions, even when performed carefully. One idea to augment the sensitivity of examination is the addition of contrast agents. The contrast agents for which there is the most clinical data over the last 40 to 50 years, include, but are not limited, to: methylene blue, acetic acid, Lugol's iodine, Toluidine blue, Cresyl violet, hypertonic saline, and indigo carmine. All of these agents are applied topically to the epithelium, sometimes in combination with each other.

How are these contrast agents thought to work? Methylene blue stains actively absorbing cells and has been used in the oral cavity and gastrointestinal tract for many years. Acetic acid causes denaturation of cytoplasmic proteins and has been used in scoping the vulva, vagina, and cervix as well as the esophagus. Lugol's iodine has an affinity to glycogen in nonkeratinized epithelium and has established use in the esophagus and cervix. Toluidine blue binds to cellular nuclei and has been used in the oral cavity and esophagus to identify areas of increased nuclear size and perhaps activity. Hypertonic saline has been used in the cervix as a contrast agent to enhance cytoplasmic size so that nuclear/cytoplasmic ratios could be calculated using the confocal microscope. It did

**Table I.** Optical technologies undergoing technology assessment for their role in cancer screening and detection categorized by their capacity to visualize large fields of view, their use in a probe that makes tissue contact, their resolution, biologic basis, relative cost, and current stage of technology assessment and commercialization.

| Modality | Large Field of View | Small Field of View Using a Contact Probe | Resolution | Biology | Cost | Technology Assessment |
|---|---|---|---|---|---|---|
| Fluorescence spectroscopy | Yes | Yes | Subcellular | Measuring the biochemistry of tissue in the epithelium and stroma | Low | Evaluated in randomized clinical trials and commercialized for use in several organ sites |
| Reflectance spectroscopy | Yes | Yes | Subcellular | Measuring hemoglobin saturation in the stroma | Low | Technical efficacy studies underway and already commercialized |
| Elastic scattering or diffuse reflectance spectroscopy | No | Yes | 10 $\mu$m | Measuring white light reflected back from chromatin in the cells of the epithelium | Low | Technical efficacy studies underway in many organ sites |
| Optical coherence tomography | No | Yes | 1–10 $\mu$m | Measuring the backscatter of light across the epithelium and stroma | High | Commercialized for the eye and under technical efficacy studies for other organ sites |
| In vivo confocal microscopy | No | Yes | 1 $\mu$m | Measures nuclei in both the epithelium and stroma | High | Commercialized and technical efficacy studies by industry and several research groups underway |

not offer an advantage over acetic acid. Cresyl violet has been used as a topical antimicrobial agent and as a stain for abnormal areas in the gastrointestinal tract. Indigo carmine is a dye that can highlight irregularities in the mucosa and has been studied principally in the gastrointestinal tract. Many of these contrast agents require that mucous be washed away before use and/or require saline washes after application to optimize staining. Most unfortunately, there are few trials in which the investigators used the same methodology for application, the same concentrations of contrast agents, or even in the same organ site, so that the quantification of the addition of contrast agents is difficult to measure.[36] In summary, no contrast agent has definitively improved the diagnosis of oral cancer.

Several research groups have been working on the area of optimizing diagnosis of oral cancer with the addition of optical technologies. Fluorescence and reflectance spectroscopy have been shown to increase the sensitivity and specificity of diagnosis when using a point probe. Perhaps a better strategy is a device that had both a wide field of view to increase the sensitivity of diagnosis combined with one of several point probe technologies that would provide measurement of specific sites to increase the specificity of diagnosis. **Table 1** shows the emerging technologies being evaluated.

White light examination has always been confounded by 2 conditions that mimic the biological phenomenon that occur during carcinogenesis: inflammation and healing. Common confounders to white light examination in the oral cavity are abrasions, trauma, viral infections, burns, and others. These are all more common in incidence than oral cancer. Many of these might be excluded at both 545 and 405 nm (that is found to be falsely positive or falsely negative). The superficial vasculature associated with inflammatory conditions and with healing should show blood vessels that decrease in size as they grow, not the atypical findings seen in neoplasia. In parallel, at 405 nm, there may be neovascularization associated with healing, but the stromal changes should be confined to the size of the injury. Thus, 2 visits using the fluorescence, in addition to white light examination, might sort out who really needs to be referred for a biopsy.

New solutions are needed for cancers like those in the oral cavity that are rare, but for which the treatments are morbid and the mortality is high. Optical technologies may provide a second level of testing in this area of need. It is said that it takes about 17 years before technologies diffuse into routine practice.[37] Research in fluorescence and reflectance spectroscopy has been carried on intensely for >20 years. Perhaps these devices will prove to be what we hope they will and answer the need for increased sensitivity and specificity, leading to higher positive and negative predictive values.

## CONCLUSIONS

Optical spectroscopy is a promising technology for the diagnosis of oral neoplasia. The conclusion of several ongoing clinical trials and an eventual randomized Phase III clinical trial will provide definitive findings that sensitivity is or is not increased over comprehensive white light examination.

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## CONFLICTS OF INTEREST

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