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Low Cost Optical Imaging Systems for Early Detection of Oral Cancer

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ABSTRACT

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Optical imaging has the potential to improve early detection of oral cancer. Reflectance and fluorescence based optical devices have demonstrated improved sensitivity and specificity compared to conventional visual oral examination. Although these devices are increasingly used as clinical tools in developed countries, they are a less practical solution in low-resource settings as the cost of these devices is relatively high, their portability is limited, and results from them are often subjective. This dissertation focuses on development of optical imaging platforms that specifically addresses these challenges and can be used to aid in screening and detection of oral pre-cancers.

The first part of this dissertation describes the construction and evaluation of a macroscopic imaging system with multi-modal imaging capability. This system can be used to screen the surface area of oral tissue at risk to detect abnormal sites. It is low-cost, portable and battery powered, which is ideal for screening and detection of oral cancer in high-risk populations in low-resource and remote settings. The system was evaluated in a clinical study in India and results from the study were used to develop a computational algorithm for objective interpretation of images from the system. In addition, this system was used to characterize optical properties of pathological conditions that are population specific. Results from this trial system are promising and show that normal oral sites can be differentiated from high-risk and cancerous sites with
high sensitivity and specificity. Although results show that sites with low-risk can be differentiated from normal tissue using this system, the sample size of the low-risk measurements is relatively small and they can not be differentiated from high-risk and cancerous tissue.

The second part of this dissertation involves developing a low-cost and simple microscopic system that is capable of high-resolution imaging for early detection of cancer. It was demonstrated that the ‘optical-sectioning’ concept of structured illumination can be integrated with optically active exogenous contrast agents for high-resolution molecular-specific imaging of pre-cancer. Finally, this dissertation also incorporates evaluation of a multi-modal miniature microscope (4M) device developed based on structured illumination. Results from the system show that the device is capable of high-resolution imaging and can be used with molecular-specific contrast agents for detection of cancer and its precursors.
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Chapter 1

Introduction

1.1 Motivation

Oral cancer is a major global health problem. It is estimated that more than 400,000 new cases of oral cancer will be diagnosed in 2008 worldwide making it the sixth most common malignancy in the world\textsuperscript{1}. Oral cancer disproportionately impacts patients in developing countries. According to the World Health Organization (WHO), of the estimated 145,500 annual deaths from oral cancer worldwide, nearly two-thirds of them occur in developing countries\textsuperscript{2,3}. In South Asian countries oral cancer is one of the most common cancers with incidence rate reported as high as 13 per 100,000 per year\textsuperscript{4}. The mortality rates in south Asia are almost twice as global rates, making it an important public health problem for this region\textsuperscript{5}.

The screening of patients for signs of oral cancer and precancerous lesions has relied upon conventional oral examination (COE). However, there are several drawbacks to this approach resulting in poor survival rate for oral cancer patients. First, oral cancer is frequently associated with development of multiple tumors which are often missed during primary diagnosis and therapeutic intervention\textsuperscript{6}. Second, detection relies heavily on clinical experience at recognition of suspicious lesions with variable effectiveness\textsuperscript{7,8}. Third, there is increasing awareness that many pre-cancerous lesions are clinically occult and not visible under conventional visual and tactile examination\textsuperscript{9}. These asymptomatic lesions can be easily ignored or missed by patients and health care professionals\textsuperscript{10}. Thus, new techniques which can aid in screening and detection of changes associated with oral
pre-cancers and can be implemented in low-resource settings and developing countries would be an important contribution to global efforts to reduce oral cancer mortality and morbidity.

Recently, optical technologies have emerged as potential aid for early detection of oral cancer. Reflectance and fluorescence based optical devices have demonstrated improved sensitivity and specificity compared to conventional visual oral examination. Currently optical technologies are a less practical solution in low-resource settings because the cost of these devices is relatively high, their portability is limited, and they require a stable, high-voltage power supply. This dissertation focuses on development of optical imaging platforms that specifically addresses these challenges and can be used as an aid in screening and detection of oral pre-cancers.

1.2 Specific Aims

My research goal is focused on developing optical technologies that are low-cost and can aid in screening and detection of oral cancer in remote settings with limited resources and facilities. Specifically, I have worked on two multi-modal imaging platforms to meet this goal: 1) a widefield system with low-resolution imaging capability and 2) a microscopic system with high-resolution imaging capability. The former is designed to screen the surface area of oral tissue at risk to detect abnormal sites with high sensitivity while the later is designed to detect molecular changes in tissue to achieve high diagnostic specificity. In addition to the above two imaging platforms, I have evaluated a miniature microscope designed by our collaborators at the University of Arizona with various biological specimens including tissues from oral cavity. My research goals of this Ph.D. thesis were as follows:
1. Develop a low-cost, multi-modal, portable widefield imaging system for screening and early detection of oral cancer.

2. Evaluate the developed optical system in a high-risk population where oral cancer is prevalent.

3. Implement a high-resolution microscopic imaging technique involving structured-illumination on a conventional microscope for molecular imaging of pre-cancer with contrast agents.

4. Evaluate imaging performance of a miniature micro-endoscope for high resolution imaging of biological specimens including pre-cancers.

1.3 DISSERTATION OVERVIEW

In this dissertation, Chapter 2 describes background information on epithelial precancer, fluorescence and reflectance imaging principles for early detection of cancer, and the concept of high-resolution imaging with structured illumination. Chapter 3 describes the design and construction of a low-cost, portable widefield imaging system with presentation of a brief set of clinical data. Chapter 4 presents evaluation of the developed portable screening system at Tata Memorial Cancer Hospital in Mumbai, India. Chapter 5 describes implementation of structured-illumination technique on a conventional bench-top microscope for high resolution molecular-specific imaging of pre-cancer. Chapter 6 presents the imaging results of a multi-modal miniature microscope (4M) from various biological specimens. Finally, Chapter 7 presents summary and conclusion of this research.
Chapter 2

Background

This chapter contains a review of the material necessary to understand the pathophysiology of oral cancer and the rationale behind optical imaging for its early detection. First, I describe the clinical significance of oral cancer and the need for early detection especially in developing countries and present an overview of the pathology of oral cancer. Second, I present a discussion of reflectance and fluorescence optical imaging principles for early detection of oral cancer. Third, I describe the imaging concept of structured-illumination and how this technique can be used together with molecular-specific contrast agents to obtain high resolution images of the molecular and morphological changes associated with oral pre-cancer.

2.1 Early Detection of Oral Cancer

Oral cancer accounts for 2% to 3% of all malignancies and has one of the lowest five year survival-rates of all major cancers. In the U.S., the population-based five-year survival rate for patients with oral cancer is approximately 54%.1 If diagnosed early, while the disease is still localized, the survival rate increases to more than 80%.1 However, for the vast majority of patients, the disease is diagnosed late, when oral cancer is at an advanced stage with regional and distant metastases. Thus, the five-year survival rate seldom exceeds 42% for those patients with regional disease and 19% for those who have disease with distant metastases.29

In developing countries, the five-year survival rate is strikingly lower compared to developed countries. For example, the average five-year survival rate for oral cancer is
only 30% in selected developing countries compared to 54% in the developed countries.\textsuperscript{30} Furthermore, even when the disease is at a localized stage and chances of survival is high, that survival rate is only 60% for developing countries as compared to 80% for the U.S and other developed countries. Figure 2.1 compares five year survival rate at different stages of oral cancer for the U.S. and India.

![Bar chart comparing five-year survival rates of oral cancer in the U.S. and India](image)

**Figure 2.1.** Five-year survival rate of oral cancer in the U.S. and India\textsuperscript{30}.

The key to improving survival of oral cancer is to improve screening and early detection. Recently, one large trial conducted in southern India examined the impact of screening on detection of oral cancer in a high-risk population vs. the general population. The study concluded that screening for oral cancer in high-risk population could reduce 37,000 deaths each year due to the disease.\textsuperscript{3} It also found that screening is less effective in the general population due to the large number of false positive referrals when screening with conventional oral examination (COE). Thus, new techniques which can aid screening and early detection of oral cancer effectively and can be implemented in
low-resource settings (i.e. developing countries) would be an important contribution to global efforts to reduce oral cancer mortality and morbidity.

2.2 PATHOLOGY OF ORAL CANCER

The oral cavity consists of multiple anatomic sites including the buccal mucosa, lips, palate, and the floor of mouth as shown in Figure 2.2a. The entire oral cavity is lined by stratified squamous epithelium which is divided into two main broad types. The masticatory epithelium covers the surfaces which are involved in processing of food (tongue, gingivae and hard palate). This type of epithelium is keratinized to different degrees depending on the extent of physical forces exerted. Lining epithelium is non-keratinized stratified squamous which covers the remaining surfaces including the buccal mucosa, lateral border of tongue, lips and the floor of mouth. Underlying the epithelium is connective tissue or stroma composed primarily of fibrous proteins such as collagen and elastin but also including fat cells, cartilage and blood vessels. In between the stroma and the epithelium lies the basal cell layer and the basal lamina or basement membrane. Basal cells are responsible for cell division and production. These cells are smaller than the epithelial cells and are cuboidal or columnar in shape as shown in Figure 2.2b. As the basal cells continue to divide, they are pushed up towards the top of epithelium displacing the older cells at the surface.
Oral squamous cell carcinoma (OSCC) originates in the epithelium.\textsuperscript{32} As normal healthy tissue comes in contact with carcinogens from tobacco, ionizing radiation, infectious organisms and other environmental toxins, changes that are precursor to OSCC can develop in the epithelium.\textsuperscript{33} Over time, the accumulation of genetic damage can cause epithelial cells to undergo neoplastic transformation resulting in the development of intraepithelial neoplasia. Ten or more years can pass between the exposure and initiation of the disease in the epithelium and the external appearance of a tumor.\textsuperscript{34-35}

Intraepithelial neoplasia begins at the basal layer of the epithelium. The loss in the cellular uniformity and architectural orientation is known as dysplasia.\textsuperscript{32} When the abnormal cells occupy approximately the lower one-third of the epithelium, the tissue is known as mildly dysplastic. As the cells continue to divide and abnormal cells occupy about one-half of the epithelium, the tissue becomes moderately dysplastic. Finally, when the entire epithelium is full of abnormal cells, the tissue is classified as carcinomain situ. At this point, the cells can invade the basement membrane and metastasize to other
areas of body resulting in invasive cancer. This dysplasia-to-carcinoma progression sequence is illustrated in Figure 2.3.

Figure 2.3. Illustration of cancer progression in an epithelial tissue at various stages.

During neoplastic progression, oral epithelial tissue undergoes various morphological and biochemical changes both at microscopic and at macroscopic level. Morphological changes of tissue at microscopic level include loss of polarity of basal cells, enlargement of cellular nuclei, variations in nuclei size and shape, and increased nuclei-cytoplasmic ratio.\textsuperscript{32,36-37} In addition, structural changes such as thickening of the epithelium, neo-vascularization, and degradation of protein filaments and collagen cross-links in the stroma are also common.\textsuperscript{36-37} Biochemical changes include changes in concentration of certain biomolecules including the cofactor reduced nicotinamide adenine dinucleotide (NADH), flavins, collagen, elastin and porphyrins.\textsuperscript{37-40} Neoplastic progression also accompanies molecular changes and overexpression of certain
biomarkers such as epidermal growth factor receptor (EGFR), matrix metalloproteinase (MMPs), and vascular endothelial growth factor (VEGF).\textsuperscript{37-43}

Neoplastic progression in the oral cavity is also generally preceded by premalignant lesions or conditions that are visible macroscopically. Most common of these conditions include erythroplakia, leukoplakia, lichen planus, ulcer and oral submucous fibrosis. Figure 2.4 shows some of these premalignant conditions. Each condition has different morphological representation, and their epidemiology and etiological factors may vary from one specific population to the other. For example, oral submucous fibrosis is a rare premalignant condition among western population but it is dominant in south Asia. Its etiology is mostly attributed to chewing of betel leaves and areca nuts that have extremely high carcinogenic potential.\textsuperscript{6}
Figure 2.4. Images of common premalignant conditions in the oral cavity.
Oral erythroplakia is defined as red lesion of the oral cavity that can not be removed and is not attributable to a specific cause. Erythroplakia has the highest malignant transformation rate compared to all other mucosal lesions varying from 14% to 50%. Up to 50% of these lesions are invasive OSCC, 40% are carcinoma in situ, and 9% have mild-to-moderate dysplasia. It can be accompanied by white patches as in leukoplakia in which case the lesion is called erythroleukoplakia. Leukoplakia is defined as any white patch or plaque that cannot be removed and is not attributable to a specific cause. Malignant transformation of leukoplakia varies between 3.6% to 17.5%. Leukoplakia that occurs on the floor of mouth and on the tongue has a higher rate of malignant transformation. One aggressive form of leukoplakia is verrucous leukoplakia which begins as a simple hyperkeratosis and progress to become multifocal and proliferative with wart-like, verrucal and exophytic feature. Verrucous leukoplakia has been reported to have 70.3% malignant transformation to verrucous carcinoma and has high recurrence rate after treatment. Lichen planus appears as patches of fine white lines and dots which usually do not cause problems. The status of lichen planus as a premalignant condition is often disputed. Several studies have shown that the transformation rate of lichen planus to OSCC is between .04% to 1.74%. However, severe forms of oral lichen planus can cause painful sores and ulcers in the mouth potentially leading to malignant transformation.

Oral sub-mucous fibrosis (OSF) is a premalignant condition that is population specific to south Asia with potential risk of malignant transformation. OSF is a chronic, progressive, and irreversible disorder that can affect any part of the oral cavity. Clinically, it is characterized by paleness of oral mucosa, oral ulceration, a burning
sensation in the presence of spicy food, hardening of the tissue and presence of fibrous bands.\textsuperscript{54} The fibrosis also leads to gradual onset of inability to open the mouth and protrusion of tongue, difficulty in mastication, speech, and swallowing with pain in the throat and ears. The malignant transformation is estimated to be between 3\% and 19\%.\textsuperscript{55} Another oral condition common in these populations is melanosis. However, unlike OSF, melanosis is considered benign and rarely associated with malignant transformation.\textsuperscript{56} Melanosis consists of dark, pigmented patches on the inside of the oral cavity induced by tobacco use. The common sites for melanosis are gingiva, buccal mucosa and tongue. It is caused by increased production of melanin to provide a biologic defence against noxious agents present in tobacco and occurs up to 21.5\% of smokers and usually disappears within three years of smoking cessation.\textsuperscript{54}

The malignant transformation rate for the above oral premalignant conditions also differs according to lesion characteristics and their location in the oral cavity. Lesions of the floor of the mouth, tongue, and lower lip are associated with higher rates of dysplasia and malignant transformation.\textsuperscript{23} Table 2.1 show most common locations of oral cancer and the corresponding incidence rate for each location.

<table>
<thead>
<tr>
<th>Location</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tongue</td>
<td>30%</td>
</tr>
<tr>
<td>Lip</td>
<td>17%</td>
</tr>
<tr>
<td>Floor of Mouth</td>
<td>14%</td>
</tr>
<tr>
<td>Gingivae &amp; Others</td>
<td>26%</td>
</tr>
</tbody>
</table>
2.3 **OPTICAL IMAGING FOR EARLY DETECTION OF CANCER**

Optical imaging technologies have been demonstrated to aid in detection oral neoplastic lesions with high sensitivity and specificity.\(^{57-60}\) The advantages of optical imaging over other imaging modalities include they are inexpensive, robust, portable and can provide near real-time diagnostic information either non-invasively or minimally invasively. Optical imaging relies on the interaction between light and tissue to detect early changes associated with the dysplasia-to-carcinoma sequence. The morphological and biochemical changes that accompany neoplastic progression affect the light absorption, scattering and fluorescence properties of tissue. These changes can be detected with a number of reflectance and fluorescence optical imaging techniques including wide-field, confocal and structured-illumination system. The reflectance-based imaging techniques rely on the variation of index of refraction among the cellular and tissue compositions, which absorb and scatter light differentially to produce image contrast. Fluorescence-based imaging systems, on the other hand, rely on certain absorbers and fluorophores in tissue to produce image contrast. The intensity and color of the fluorescence also provide important biochemical information about the tissue including its composition, presence or absence of certain fluorophores as well as its metabolic activities. Beside the native sources of contrast present in cells and tissue, certain exogenous contrast agents can also be introduced for optical imaging. These contrast agents generally have higher scattering properties and quantum efficiency for reflectance and fluorescence imaging respectively, and yield higher signal-to-background contrast than native sources.
2.3.1 Fluorescence Imaging

Fluorescence is the process by which excitation light evokes emission of light of a different wavelength. The excitation light is usually in the near-UV to visible range and the emitted fluorescence light is in the visible to near-infrared range. Fluorescence is the result of a process that occurs in certain molecules called fluorophores in which photon energy supplied by the excitation light source is absorbed by the molecule, creating an excited electronic singlet state. The fluorophores remain in the excited state for a short period of time and return to the ground state. When the fluorophores return to the ground state, photons with energy lower than the excited photons are emitted as fluorescence.

There are many endogenous fluorophores in tissue with distinct optical properties. Figure 2.5 shows excitation and emission spectra of important fluorophores that are reported to be associated with neoplastic development including NADH, FAD, tryptophan, porphyrin, collagen and elastin. The NADH or reduced form of nicotinamide adenine dinucleotide and the FAD or oxidized form of flavin adenine dinucleotide, are indicators of cellular metabolism and are located predominantly within the mitochondria of cells. Thus, NADH and FAD-induced auto-fluorescence originates from the epithelial layer of tissue where cells are metabolically active and go through continuous division. Maximum NADH fluorescence occurs at 340-nm excitation and 450-nm emission wavelengths while that of FAD occurs at 450-nm excitation and 530-nm emission wavelengths. Drezek et al and Pavlova et al found that NADH fluorescence increases with neoplastic progression while that of FAD decreases.
Figure 2.5. Excitation (top) and emission spectra (bottom) of some native fluorophores in tissue associated with neoplasia. Adopted from ref. 62.
Another source of fluorescence in the epithelial tissue is collagen and elastin, which are fibrous proteins that predominantly make up the stroma under the basement membrane. Together, they provide a strong supporting structure for the epithelial tissue. Collagen and elastin autofluorescence are associated with their enzymatically activated crosslinks. Collagen has maximum fluorescence at 340-nm excitation and 390-nm emission wavelengths while that of elastin occurs at 350-nm excitation and 420-nm emission wavelengths. At near-UV to visible wavelength excitation of healthy tissue, epithelial autofluorescence is mostly attributed to collagen and elastin crosslinks. However, collagen- and elastin-related autofluorescence in premalignant lesions have been reported to decrease significantly possibly due to the breakdown of crosslinks in collagen and elastin. The increased thickness of epithelial layer due to neoplastic progression is also considered a likely cause for this decrease in fluorescence. Other important factors that contribute or affect auto-fluorescence in tissue include porphyrin and hemoglobin. Porphyrin has a distinctive fluorescence characteristic with a maximum intensity at 400-nm excitation and 635-nm emission. The affinity of porphyrin to malignant tissue has been much studied and is somewhat debatable. A most common theory is that porphyrin-like fluorescence is a product of microbial porphyrin synthesis, and therefore, is only limited to necrotic surface of exulcerated tumors. Another important factor that affects tissue autofluorescence is hemoglobin. The amount of blood vessels present in certain tissues has shown to influence the autofluorescence characteristic of the tissues. Hemoglobin in blood has a strong light absorption property, and absorbs both excitation and emitted fluorescence light. In neoplastic tissue, angiogenesis results in an increased microvascular density and the amount of blood in the
tissue. This increase in the concentration of blood in tissue decreases the overall tissue autofluorescence.

2.3.2 Reflectance Imaging

Reflectance imaging involves detection of the same wavelength(s) of emitted light that are illuminated on the tissue. Reflectance-based techniques rely on absorbers and scatterers of light in tissue to produce morphological and biochemical information. Most common sources of reflectance contrast that are pertinent to neoplastic tissue include cellular nuclei and their chromatin content, tissue morphology and architecture, and hemoglobin. The nuclear size and nuclei-to-cytoplasmic ratio increases with neoplastic progression. Drezek et al. have shown through modeling and experimentation that this increase in nuclear size and overall nuclei-to-cytoplasmic ratio increases the light scattering from the epithelium. The scattering properties of the stromal layer underlying the epithelium also change with neoplastic progression. It is believed that the morphological structure of collagen and elastin changes and the volume fraction of the fibers decreases as dysplasia develops, leading to decreased scattering from the stroma. Hemoglobin plays an important role in absorption of light in the stromal layer. Increase in hemoglobin concentration due to increased microvascular density and angiogenesis increase the light absorption properties of tissue.
**Figure 2.6.** Schematic of contrast agents (indicated with yellow spheres) bound to epidermal growth factor receptors (EGFR) on the cell membrane. Adapted from ref. 70.

### 2.4.3 Imaging with Contrast Agents

Molecular specific contrast agents are exogenous fluorophores or contrast materials that can be used to highlight specific molecules of interest and probe their presence in cells and tissues. They enhance the contrast of target molecules that have either poor or no contrast. A number of contrast agents are currently being studied for optical imaging that can highlight up-regulated proteins in neoplastic cells and tissues such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) receptor and matrix metalloproteinase (MMPs). The contrast agents can be divided into two categories according to imaging modalities: reflectance and fluorescence contrast agents. Reflectance contrast agents such as gold and silver nanoparticles are used to probe molecules in reflectance imaging while fluorescence contrast agents such as quantum dots and dyes are used for fluorescence imaging. Although there are many
more contrast agents, my discussion in this dissertation is limited to only two of these contrast agents: gold nanoparticles and quantum dots.

Gold nanoparticles are colloidal gold nanospheres made from the reduction of auric acid with sodium citrate.\textsuperscript{71} They have the ability to scatter light very strongly over a narrow band wavelength due to the excitation of surface plasmon resonance and collective oscillation of electrons in electric field.\textsuperscript{71} They scatter light in the visible and near IR wavelength which can be easily detected using reflectance imaging systems. Sokolov et al. have demonstrated the use of conjugated gold nanoparticles with monoclonal antibodies to successfully image cancerous cells and tissues in reflectance mode.\textsuperscript{72} Imaging with a reflectance confocal microscope, the authors have demonstrated that the anti-EGFR gold nanoparticle conjugates label only premalignant cells and tissues. They found that the strong light-scattering from the gold nanoparticles bound to the cell membrane of the premalignant cells and tissues allows one to see cellular morphology clearly and probe the presence of cancer biomarker EGFR.

Another type of contrast agent that has recently been widely used for optical imaging applications is quantum dots. Quantum dots are semiconductor nanocrystals with a quantum-confinement property that enables them to emit different colors of light.\textsuperscript{73} Thus, they are used for fluorescence imaging applications. Quantum dots (QDs) are bright with broad absorption property in the blue region of visible spectrum while they have narrow emission property from the visible to the near IR region of the spectrum. Due to the large separation between their excitation and the emission wavelengths or Stokes' shift, quantum dots result in much higher signal-to-background ratio than conventional fluorescence dyes or contrast agents. Another advantage of the quantum
dot is they are not prone to photo-bleaching. A number of studies have shown the potential of using quantum dots for diagnosis of cancer.\textsuperscript{73-75}

2.4 STRUCTURED-ILLUMINATION

In any imaging application, resolution of the imaging system plays a vital role in differentiating morphological features in cells and tissue. Molecular-imaging, in particular, requires reflectance and fluorescence imaging systems that has the ability to delineate molecular features both in lateral and in axial direction. Although the lateral resolution in a conventional wide-field microscope can provide fine details of cells and tissue, its axial resolution is often poor. Confocal microscopy is generally used to obtain high resolution images both in lateral and in axial direction. One drawback, however, is it is much more complex and expensive. The structured illumination technique is a simple alternative to complex confocal microscopy technique that improves the axial resolution of a conventional widefield imaging system.\textsuperscript{76} It was first proposed by Wilson \textit{et al.} for a widefield microscope and later was extended to macroscopic imaging systems.\textsuperscript{77-80} The authors demonstrated that the axial resolution of a conventional microscope could be dramatically improved to approach that of a confocal microscope through the optical-sectioning of imaging object using structured grid patterns and subsequent mathematical processing of acquired images.

'Optical sectioning' refers to faithful imaging of objects that are within the depth-of-field of a microscope without any out-of-focus light. In a conventional widefield microscope, objects in the image plane include features that are both within and outside of the depth-of-field of the microscope, resulting in image 'blurring'. Structured illumination technique modifies the illumination path of a widefield microscope so that
the out-of-focus contributions can be differentiated from the in-focus components, producing optically sectioned images similar to confocal microscopes. In mathematical terms, in a conventional widefield microscope all but the zero (a.k.a. DC) spatial frequency components attenuate with defocus. The zero spatial frequency corresponds to unwanted background and noise generated from regions outside the depth-of-field of the microscope and causes the image 'blurring'. This out-of-focus light reduces contrast and affects details that are within the system's depth-of-field. The key to generating 'optical sectioned' images lies in that all spatial frequencies including the zero must be attenuated with the defocus of the optical system. To accomplish this, a single-spatial-frequency grid pattern is projected onto the focal plane of a microscope through its conjugate focal plane. The one dimensional grid pattern can be represented in optical coordinates \((t, w)\) as

\[
S(t_0, w_0) = l + m \cos(\hat{v}t_0 + \varphi_0), \quad \text{Eq. [1]}
\]

where \(\varphi\) is arbitrary phase and \(\hat{v}\) is the normalized spatial-frequency related to actual spatial frequency, \(v\), by \(\hat{v} = v \cdot \beta \cdot \lambda / NA; \) \(\beta\) is the magnification between grid pattern and object plane, \(NA\) is the numerical aperture of the objective lens and \(\lambda\) is the wavelength of light. The projection of the grid pattern on the object plane is such that all spatial frequencies in the object plane except the zero frequency will have 'optical signatures' on them and the resulting image can then be written as

\[
I(t, w) = I_0 + I_c \cos \hat{v}t_0 + I_s \sin \hat{v}t_0. \quad \text{Eq. [2]}
\]

Here, \(I_0\) represents conventional wide-field image with both in- and out-of-focus components, \(I_c\) and \(I_s\) represents images that are due to grid pattern of form \(m \cos (\hat{v}t_0)\) and \(m \sin (\hat{v}t_0)\). From Eq 2, we notice that only the non-zero spatial frequency
components or objects within the depth-of-field have grid pattern while zero spatial frequency components or objects outside the depth-of-field does not have any pattern.

\[ I_1 = I_0 + I_c \]  
Eq. [3]

\[ I_2 = I_0 - \frac{1}{2} I_c + \frac{\sqrt{3}}{2} I_s \]  
Eq. [4]

\[ I_3 = I_0 - \frac{1}{2} I_c - \frac{\sqrt{3}}{2} I_s \]  
Eq. [5]

\[ I_p = \left[ (I_1 - I_2)^2 + (II - I_3)^2 + (I_2 - I_3)^2 \right]^{1/2} \]  
Eq. [6]

Therefore, in order to recover non-zero spatial frequency components without the grid patterns, at least three images (\( I_1, I_2, \) and \( I_3 \)) are needed with the grid pattern shifted by a third of a cycle or spatial phase of the pattern each time (\( \varphi_0=0, \varphi_0=2\pi/3 \) and \( \varphi_0=4\pi/3 \)). Finally, the optically sectioned image (\( I_p \)) can be generated by applying the simple square root method. Since the zero spatial frequency components do not have any grid pattern, they will simply cancel out. Figure 2.7 shows a graphical representation of obtaining optical sectioning using structured illumination.

![Figure 2.7. A schematic of structured illumination technique.](image-url)
Wilson et al. have demonstrated high-resolution optical sectioning ability of structured illumination both in reflectance and in fluorescence imaging mode. In fluorescence mode, the technique was demonstrated with lily pollen grain and COS-7 kidney cells. The group also demonstrated real-time three-dimensional imaging of wrist watch mechanism in reflectance mode.

One fundamental drawback to structured illumination is that in order for optical sectioning to produce high resolution images, sufficient contrast must be present in the sample. The endogenous sources of contrast in many biological samples including cells and tissues do not provide the contrast needed for structured illumination due to their low scattering or quantum yield property. The useful in-focus contrast information can be limited to even as low as two gray levels of the camera, and standard algorithms are not capable of effectively reconstructing images of the in-focus section under these conditions. Furthermore, reflectance imaging of tissue is generally sensitive only to non-specific differences in refractive index, and provides rather limited information about the range of molecular changes associated with patho-physiology. The magnitude of the refractive index difference between the nucleus and cytoplasm in epithelial cells is small ($\Delta n \sim 0.02$), thus the signal from the optical section is small compared to the multiply-scattered out-of-focus light. Simple, non-specific contrast agents such as acetic acid (vinegar) are used routinely in clinical cancer detection and can enhance nuclear backscattering several fold, but the magnitude of the in-focus signal compared to the out-of-focus signal is still small. Similarly, the endogenous fluorophores present in tissue have very low quantum efficiency. To image these samples, exogenous contrast agents such as gold nanoparticles and quantum dots or dyes must be used. Thus, integrating
structured illumination with contrast agents can provide a simple way of obtaining high-resolution images for detection of cancer and precancer. This concept of integrating structured illumination with molecular specific contrast for high resolution imaging is described in detail in Chapter 5.
Chapter 3

Low-Cost, Multi-Modal, Portable Screening System for Early Detection of Oral Cancer.

3.1 INTRODUCTION

Oral cancer is an important global health problem that disproportionately impacts patients in developing countries. According to the World Health Organization (WHO), the worldwide annual incidence for oral cancer exceeds 267,000 new cases with an estimated 145,500 deaths, nearly two-thirds of which occur in developing countries. Overall survival rates for oral cancer in developing countries are low and there has been little improvement over the past three decades. In the vast majority of cases today, oral cancer is diagnosed at an advanced stage, when it requires more aggressive treatment and results in poor survival and increased morbidity. However, one large screening trial conducted in southern India demonstrated that early detection of oral cancer in high risk populations can reduce mortality. Thus, new techniques which can aid diagnosis of changes associated with oral cancer at an early stage and can be implemented in low-resource settings would be an important contribution to global efforts to reduce oral cancer mortality and morbidity.

The standard method for screening and detection of oral neoplasia is visual inspection of oral cavity under white light. Unfortunately, the clinical appearance of oral

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cancers at an early stage can be difficult to differentiate from nonspecific inflammation and irritation under white light examination. Fluorescence imaging has been shown to be an effective alternative method for screening and diagnosis of pre-cancers in several organ sites including oral cavity, uterine cervix, lung and skin everal groups including Betz et al, Onizawa et al, Paczona et al and Sivstun et al have shown that examining the oral cavity under a fluorescence excitation light source can overcome some of the detection limitations associated with standard white light examination. Lane et al recently proposed a simple hand-held device for direct visualization of tissue autofluorescence above 480nm using a metal halide mercury lamp with excitation wavelengths between 360-460nm. The device is currently approved for medical use by the Food and Drug Administration (FDA) in the U.S.

Although previous fluorescence imaging devices have shown high sensitivity and specificity for detecting abnormalities in the oral cavity, their use has been mainly limited to medical facilities in developed countries. They are a less practical solution for mass screening of high-risk populations in low-resource settings as the cost of these devices is relatively high, their portability is limited, and they require a stable, high-voltage power supply. Furthermore, it is difficult to use these devices to perform traditional white light examination, which may prevent clinicians from obtaining clinical impressions they are accustomed to observing. In this chapter, we present a simple yet robust optical system that can be used for both white light reflectance examination and fluorescence imaging. The head-mounted device presented here can be used for direct visualization by the health care provider, as well as for digital acquisition of images of the oral cavity. This inexpensive and portable device utilizes light-emitting diodes (LED) as its illumination.
source and can be powered by a compact lithium-ion battery. This approach not only makes the device low maintenance and affordable but also makes it usable in low resource settings where a stable source of electrical power may not be available. Altogether, the features of the optical device make it easily translatable to developing countries for screening oral cancer.

3.2 INSTRUMENTATION

A schematic diagram and picture of the optical device, named the portable screening system (PS2 Oral), is shown in Fig 3.1. The system is designed for direct visualization and digital imaging for wide-field macroscopic inspection of the oral cavity. It has multi-modal imaging capabilities including white-light reflectance, orthogonal polarization and fluorescence. There are three principal components to the system: illumination, detection and system integration. Detail attention was given in selection of each of the components such that the system could be adaptable to conditions in remote locations and low-resource settings. In this section, we discuss each of the components in detail.

3.2.1 Illumination

Ideal imaging system requires light sources that have very uniform fields, excellent control of relative intensity and known spectral contents. Traditionally, halogen lamps have been used for white light and reflectance imaging due to their uniform spectral characteristics in the visible-near IR wavelengths. For fluorescence imaging applications, high power arc lamps such as xenon and mercury lamps are commonly used. The arc lamps provide high irradiance required to excite endogenous and exogenous fluorophores, and generate fluorescence. However, there are several
Figure 3.1. Schematic diagram (top) and picture (bottom) of the portable screening system for oral cancer.
drawbacks to these conventional light sources that limit the use of existing fluorescence imaging devices such as the VELScope in only resourceful settings such as medical facilities in developed countries. First, the cost of the light sources can be significant due to its highly-regulated voltage supply. Second, they require high voltage power supply that is often not available in low-resource and remote settings. Third, arc lamps are inherently unstable due to fast changes in the electron density in the generated plasma and thermal runaway. Fourth, these light sources have very limited portability due to safety concerns and the weight of the sources.

An alternative to traditional reflectance and fluorescence illumination sources is light emitting diodes (LEDs). Recent advances in high performance LED technology have offered a cheap, stable and easy-to-use solution for light source that can be used in a wide variety of imaging applications. A number of commercial vendors now produce high-performance LED that provides sufficient intensity at specific wavelengths especially for fluorescence imaging applications. The advantage of LED over traditional light sources include their compact size, low power consumption, minimal heat output, fast switching and adjustable properties, high emission stability and extremely long life span. All of these advantages play a crucial role for use of our desired imaging system in low-resource and remote settings.

The use of light emitting diodes as illumination source for autofluorescence imaging of the oral cavity is new and has not been explored previously to the best of our knowledge. Most studies that have reported use of LED in fluorescence applications involves imaging with exogenous contrast agents, which have higher quantum efficiency
than endogenous fluorophores. To determine whether LED can be used as excitation light source for endogenous fluorophores such as collagen and elastin, the required minimum irradiance at tissue was investigated. Trujillo et al have reported fluorescence quantum efficiency (QE) of cervical epithelial tissue varying from 0.0006 for normal to 0.0003 for precancer at 460-nm excitation\(^8\). Based on this quantum efficiency, Benavides et al. found that a CCD camera with detection sensitivity of \(8 \times 10^{-15}\) W per pixel can detect autofluorescence with an irradiance of 1.3 mW/cm\(^2\) at the tissue with signal-to-noise ratio (SNR) of 10\(^8\). For our PS2 imaging system, a 750mW blue LED (Luxeon Royal Blue- K2) with 455-nm wavelength was considered for fluorescence illumination. This light source provided a peak irradiance of 15mW/cm\(^2\) at the center of the measurement at a working distance of 25 cm. Thus, the illumination intensity of our LED far exceeds that required for fluorescence illumination of tissue provided that a CCD with similar specifications as above is used. For reflectance illumination, a white LED (Heine USA) was used; its intensity was comparable to the conventional white lights used by health care professional for regular clinical examination of the oral cavity. Figure 3.2 shows intensity profile of the illumination light sources across the field-of-view of the PS2 system.
Figure 3.2. Intensity profile of the LED light sources in PS2 imaging system.

One important parameter for fluorescence illumination source is the excitation wavelength(s) desired for optimum emission of fluorophores. Ideally, an illumination light source should only have the excitation wavelength(s) at which the quantum efficiency for the desired fluorophore(s) has maximum emission peak. Extensive studies have been conducted by various research groups in understanding both how bio-fluorophore concentration changes with dysplastic progression and what optimal wavelengths are best for probing the changes. Utzinger et al. have shown that the optimal excitation wavelengths for detecting abnormal oral tissue are between 420 and 440 nm\(^9\). Svistun et al. extended the previous work on freshly resected oral tissue and found that best sensitivity and specificity were achieved with excitation wavelengths 400 nm and 440 nm, and emission collection between 500 and 560 nm\(^9,82\). Based on these works and others, Lane et al. used metal halide mercury lamp with excitation peak at 405-nm and 436-nm for their hand-held fluorescence device commercially available as VELScope. The Luxeon Royal Blue light emitting diode selected for the PS2 system had an excitation peak at 455nm. This is not the optimum excitation wavelength suggested in the literature. However, the selection of the PS2 fluorescence illumination source was
limited by the commercial availability of the LEDs, their maximum irradiance at tissue and their peak intensity wavelength. Figure 3.3 shows the wavelength profile of the royal blue LED used for fluorescence illumination in the PS2 system.

![Wavelength profile of PS2 LED fluorescence illumination source.](image)

**Figure 3.3.** Relative intensity vs. wavelength of PS2 LED fluorescence illumination source.

### 3.2.2 Detection

Selection of right camera is crucial in any imaging systems especially for fluorescence imaging applications. Fluorophores with low quantum efficiency requires highly sensitive detector. At the same time, the weight and size of a sensitive detector can limit its portability and usage in clinical settings. In addition to these factors, our detector selection was also limited by its power consumption rate, which must be low enough such that it can be powered by battery in remote locations. For our imaging system, we selected a head-mount color CCD camera (Prosilica EC1380C) that can be used for both reflectance and fluorescence imaging. The 1.3 megapixels camera weights only 89 grams and has a dimension of 33 mm x 46 mm x 66 mm. The CCD contains a
progressive scan chip (Sony ICX285AL, Exview HAD) with 6.45um x 6.45um size pixels and a sensitivity of 0.02 lux at 100ms exposure time. At this sensitivity, the camera can detect $1.2 \times 10^{-15}$ W per pixel. This detection sensitivity is much higher than the required specification to detect the minimum fluorescence emitted from tissue with the excitation of our light source. The power consumption of the camera was less than 3W and could be supplied by firewire interface via laptop computers for remote use. A graphical-user-interface (GUI) software was written in LabView (National Instruments) to control the camera and acquire images.

3.2.3 System Integration

In order to integrate the desired illumination and detection components discussed above, a commercially available surgical headlight system (Heine, USA Ltd) was modified. The headlight system provided a supporting frame to hold the illumination source and the detector together. The headlight system also provided a lithium-ion battery which could be used to power the illumination source of the PS2 system continuously for four hours. For visual detection, a pair of binocular loupes (Heine USA Ltd) with 2.5x magnification was purchased. The loupes had two functions: it provided magnified view of the observation sites, and served as filter holder in front of the eyes for visual observation of fluorescence emission from tissue. The binocular loupes provided a field of view (FOV) of 55 mm at a working distance (WD) of 25 cm, with a depth-of-field (DOF) of 55mm. The spatial resolution of the system was tested with a USAF resolution target and up to four lines pairs per millimeter could be resolved.

In order to record images from tissue in different modalities including fluorescence, standard white light and orthogonal polarized modes, appropriate optical
components were introduced in the optical light path. For fluorescence illumination, a
447/60 nm excitation filter (Semrock FF02-447/60) was placed in front of the blue LED
to prevent any bleed-through above 480 nm. The emitted fluorescence could be observed
or detected through 480 nm long pass emission filters (Omega Optical 480ALP). Similarly, for orthogonal polarized light reflectance imaging, a linear polarizer was
placed in front of the white LED light; an additional linear polarizer oriented at 90°
relative to the first was placed in the detection light path. This simultaneously blocks
specular reflection and allow observation of lesions and deeper tissue structures such as
microvasculature which may not exhibit high contrast under standard white light
illumination\textsuperscript{91}. The long-pass filter and polarizer in the detection path of the camera
were placed using a custom designed filter holder. This holder contains three positions to
allow different optical components to be easily interchanged during patient examinations
in different imaging modalities. The total cost of the PS2 system as assembled here was
approximately $4,500 excluding the cost of the PC. The system cost can be reduced to
$1,200 by replacing the scientific grade camera with a low cost camera which is sensitive
enough to detect autofluorescence. We estimate that the final cost could be drastically
reduced to less than $100 by eliminating the camera altogether, but this would eliminate
the capability for recording imaging data for clinical or research purposes.

3.3 Preliminary Results

The system was used to obtain images of the oral cavity from normal volunteers
and from patients suspected to have oral cancer at the UT MD Anderson Cancer Center.
Following image acquisition in consenting patients, clinically suspicious areas of tissue
were biopsied and submitted for histopathologic diagnosis. In addition, the system was
used to image the oral cavity of consenting patients at the Tata Memorial Hospital in Mumbai, India.

**Figure 3.4.** Digital images of the floor of mouth from a normal volunteer using the portable screening system: (a) fluorescence (b) standard white-light reflectance, and (c) orthogonal polarized white-light reflectance images.

Figure 3.4 shows images of the floor of mouth of a normal volunteer obtained using different imaging modalities with the portable screening system. Although the bright fluorescence signal from the teeth partially saturates the image, green autofluorescence signal from tissue is clearly visible in Fig. 3.4a. Similarly, in the standard white light image, strong specular reflection in some regions partially saturates the image and hinders observation of underlying tissue structures. In comparison, in the orthogonal-polarized image, the specular reflection is removed allowing good visualization of the sub-epithelial vasculature as shown in Fig. 3.4c.

Figure 3.5 shows images of the oral cavity from three patients obtained using the portable screening system. The top row shows images from the floor of mouth of Patient A; the arrow in the fluorescence image indicates an area with strong loss of fluorescence; in contrast, the standard white light image and the orthogonal polarized white light images of the same area showed no obvious clinical abnormalities. Both the white light images show the proposed region of tissue to be resected drawn by the surgeon based on
clinical judgment. The histo-pathology report confirmed an area with dysplasia within this region.

In addition, Figure 3.5 shows images from two patients with clinically apparent lesions. Although histopathology was not available in these patients, regions of loss of fluorescence correlate well with clinically suspicious areas. Images are shown from Patients B and C with leukoplakia in the left buccal mucosa. In both cases, there is a strong loss of fluorescence associated with the area of leukoplakia. Images from Patient B show a region of increased redness in the orthogonal polarized image just beneath the area of leukoplakia (arrows); this also corresponds to a region of decreased fluorescence. Images from the contralateral normal buccal mucosa in Patient C show homogeneous fluorescence across the tissue.
<table>
<thead>
<tr>
<th>Patient Number/Clinical Impression</th>
<th>Fluorescence image</th>
<th>Standard white light reflectance image</th>
<th>Orthogonal polarized light reflectance image</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient A:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysplasia</td>
<td></td>
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<tr>
<td><strong>Patient B:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukoplakia at left lower labial margin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Patient C:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukoplakia at left buccal mucosa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contralateral normal buccal mucosa</td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 3.5.** Images of the oral cavity from three patients obtained with the portable screening system: (left column) fluorescence images, (middle column) standard white-light reflectance images, and (right column) orthogonal polarized white-light reflectance images.
3.4 DISCUSSION

The potential of the PS2 system to help differentiate between normal and dysplastic oral tissue is demonstrated in Figs. 3.4 and 3.5. In contrast to the images from the normal volunteer, the autofluorescence signal from dysplastic tissue in Patient A shows a characteristic loss of autofluorescence. Similarly, in Patients B and C, mucosal areas with lesions clinically suspicious for dysplasia also show characteristic loss of autofluorescence. This is consistent with previous research using more costly scientific grade instruments; this loss of autofluorescence is attributed to a decrease in stromal collagen and elastin fluorescence which underlies the dysplastic epithelial tissue. Furthermore, the use of orthogonal polarization imaging reduces interfering specular reflection and enhances contrast associated with subsurface vasculature which is frequently increased in neoplastic lesions.

In summary, images obtained with the PS2 system in-vivo demonstrate that it is an attractive technology to explore for cancer screening in low-resource environments where clinical expertise is often unavailable. The device incorporates LED illumination, and can be used in both white light and fluorescence imaging modes. It is battery powered and can acquire digital images of tissue with an integrated CCD camera. These stored images can be used in longterm clinical surveillance, for telemedicine review by clinical experts, and potentially for incorporation of automated image analysis algorithms. Overall, these features, coupled with the low cost of the system, make it an attractive option to explore for oral cancer screening in low-resource settings. Further clinical trials in a controlled, prospective setting are required to evaluate the diagnostic performance of the device and to determine the degree of clinical expertise required to
use it effectively. Although the device is designed for screening for oral cancer, it can be translated to other organ sites. Furthermore, as new understandings in optical properties of normal and diseased tissue are conceived and advances in LED technologies are made, they can be easily incorporated into the PS2 system to improve the device.
Chapter 4

Evaluation of A Low-Cost, Portable Imaging System for Screening and Early Detection of Oral Cancer

4.1 Introduction

Oral cancer is a major health problem worldwide, causing over 127,000 deaths each year. With an annual incidence exceeding 274,000 cases, oral cancer ranks as one of the top ten most common malignancies. Over two-thirds of cases and three-quarters of deaths due to oral cancer occur in developing countries. In the U.S., the overall five-year-survival rate for patients with oral cancer is only 54%, one of the lowest rates of all major cancers; in developing countries, five-year survival rates drop below 30%.

Patients with early lesions have better chances for cure and less treatment associated morbidity, yet despite the easy accessibility of the mouth, most patients present with advanced tumors, when treatment is more difficult, more expensive and less successful compared to earlier interventions. Early detection of oral premalignant lesions (OPLs) and early neoplastic changes may be our best and most cost-effective means to improve survival and quality of life for oral cancer patients from all socioeconomic communities.

No satisfactory mechanism exists currently to screen and detect early neoplastic changes of the oral cavity in the general population. Possible explanations include: 1) Limited public awareness and insufficient education of health care workers about oral cancer risk factors, signs and symptoms. 2) Detection relies heavily on clinical

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1This chapter is modified from: M. Rahman, N. Ingole, D. Roblyer, V. Stepanek, R. Richards-Kortum, A. Gillenwater, S. Shastri, P. Chaturvedi; "Evaluation of A Low-Cost, Portable Imaging System for Early Detection of Oral Cancer," TBA.
experience at recognition of suspicious lesions during physical examination, with variable effectiveness.\textsuperscript{9,10} It can be difficult to distinguish OPLs from more common inflammatory conditions. 3) Practitioners and patients are reluctant to perform invasive biopsies of oral lesions which are expensive and often require referral to a specialist. 4) Some patients have field cancerization, where the entire mucosal lining that is exposed to carcinogens in alcohol and tobacco sustains damage and is at risk to develop cancer.\textsuperscript{92} In high risk patients, often the whole lining of the oral cavity is potentially premalignant, making it difficult even for experienced clinicians to know when and where to biopsy.\textsuperscript{9} The situation is even more challenging in developing countries and low-resource regions with high-risk populations, where a combination of lack of public awareness of the disease and inadequate resources and expertise for screening can result in even greater delays in diagnosis, leading to higher morbidity and mortality.

Recent advances in optical imaging have the potential to improve early detection of oral cancer and its precursors. Several groups have demonstrated that imaging systems that record the spatial distribution of tissue fluorescence at specific excitation/emission wavelength combinations can be used to survey large areas of oral cavity mucosa to non-invasively detect early changes associated with oral cancer in real-time.\textsuperscript{9,59-61,81-83} Lane presented a non-magnifying hand-held device for direct visualization of oral cavity tissue fluorescence, which is now FDA approved for clinical use and commercially available as the VELscope®.\textsuperscript{5} The system uses a metal-halide lamp with emission peaks at 405 and 436 nm to excite autofluorescence; images viewed by eye through the VELscope indicate a characteristic loss of fluorescence associated with
malignant progression. Results from 50 biopsies taken from areas with loss of fluorescence in 44 patients showed a sensitivity of 98% and specificity of 100% for discriminating normal tissue from severe dysplasia, carcinoma in situ, or invasive carcinoma, using histology as the gold standard. An important finding was the ability of fluorescence visualization to aid clinicians in identifying early neoplastic lesions that were initially missed during traditional white light examination (WLE).  

To be useful in low resource settings where the vast majority of oral cancers occur, screening aids must be affordable and must not rely on significant clinical expertise for image interpretation. The ability of fluorescence-based screening aids to differentiate precancerous lesions from benign lesions such as inflammation must also be validated in larger trials. Finally, the optical properties of other potentially confounding lesions, which may be population specific, must be characterized. For example, betel quid use is common in south Asia and is associated with a high incidence of OPLs as well as other potentially confounding oral lesions, including melanosis and oral submucous fibrosis (OSF).  

To address the need for objective image interpretation, Roblyer recently described a multimodal digital microscope to obtain digital images of oral tissue fluorescence and reflectance and evaluated the ability of objective image analysis techniques to recognize OPLs and early oral cancer. Using 405 nm excited fluorescence images, algorithms could detect OPLs and oral cancer with a sensitivity of 94% and specificity of 87% in 64 patients. The results of automated analysis of digital autofluorescence images must also be validated in larger clinical trials.
While digital image analysis can provide more objective interpretation, lower cost devices are needed for use in developing countries. To address the need for low-cost imaging, Rahman recently developed a multi-modal imaging system for screening and detection of oral cancer in high-risk populations in low-resource and remote settings. This portable, battery powered system acquires near real-time digital images of oral tissue in reflectance and fluorescence mode. In this paper, we report results of a clinical study to evaluate the ability of this device to aid in detection of oral cancer in India involving 109 subjects at high-risk for developing oral cancer. We also characterize the optical properties of two types of potentially confounding oral lesions, melanosis and oral submucous fibrosis (OSF), which are common in this region, and discuss how the presence of these lesions influences the ability of the device to accurately identify neoplastic lesions.

4.2 Materials and Methods

4.2.1 Instrumentation

The portable imaging system used in this study consisted of a modified commercial headlamp system; details of the device have been described previously. Briefly, the multi-modal imaging system uses light emitting diodes (LEDs) to illuminate the oral mucosa. For fluorescence imaging, the system has a blue LED with an excitation peak at 455 nm wavelength; for reflectance imaging, it has a white LED with an illumination range of 400 to 700 nm. Images can either be observed visually or captured digitally through a set of optical filters using an integrated, miniature charge coupled device (CCD) camera. The system is connected to a laptop via a firewire interface to
record and store the images. The portable system weights only three pounds and can be powered by a lithium-ion battery.

4.2.2 Protocol and Image Acquisition

The study was conducted at Tata Memorial Hospital (TMH) in Mumbai, India. Patients who were referred to the Cancer Prevention Clinic at Tata Memorial Hospital because of suspicious oral lesions or were waiting for head & neck surgery in the hospital ward were recruited to participate in the study. In addition to patients, normal volunteers with and without a history of using tobacco were recruited to participate in the study. The clinical study was reviewed and approved by the Hospital Ethics Committee (HEC) at TMH and the image analysis study was reviewed and approved by the Institutional Review Board at Rice University. Written informed consent was obtained from each subject enrolled in the study.

In vivo imaging measurements from subjects were obtained in the Cancer Prevention Clinic. All measurements were taken in a darkened room to avoid room light interference. The imaging system was positioned approximately 20 cm away from the subjects. Reflectance image exposure was a few milliseconds while fluorescence image exposure was approximately 500 milliseconds.

A head & neck specialist at the clinic assessed each participating patient by conducting a conventional examination of the oral cavity. Initial clinical impression of each site as normal or abnormal was noted. For each clinically abnormal site, clinical descriptors were noted, including the following categories: melanosis, lichen planus, OSF, hyperkeratosis, ulcer, leukoplakia, erythroplakia, and/or cancer. After clinical examination, digital reflectance and fluorescence images were obtained from clinically abnormal sites and contralateral clinically normal sites. Images were also obtained from
the lateral border of the tongue, the buccal mucosa, and the lip of each subject whenever these sites were accessible. A quality control check was performed on all images before further analysis. Sites with poor image quality (e.g. out-of-focus images) were excluded from analysis.

For sites with an initial clinical impression of abnormal, the white light reflectance images were reviewed by three expert observers who were blinded to the fluorescence images (NI, AG, PC). At each site, the observer noted one or more clinical descriptors for the lesion, including: melanosis, lichen planus, OSF, hyperkeratosis, ulcer, leukoplakia, erythroplakia, and/or cancer. In addition, each site was assigned a single diagnostic category of ‘Normal’, ‘Low Risk for Neoplasia’, ‘High Risk for Neoplasia’, or ‘Cancer’. Consensus clinical impression was used to determine the final diagnostic category for each site imaged; performance of algorithms based on features of digital optical image analysis are reported relative to this diagnostic category. Sites with an initial clinical impression of normal were categorized with a diagnosis of ‘Normal’. In cases where the impression of one of the expert observers differed from the other two, the diagnostic category assigned by two of the three observers was used. Measurements in which all three observers disagreed on the diagnostic category were excluded from the analysis; a total of four sites were excluded for this reason.

Histopathologic evaluation was available for 57 sites imaged. In these sites, the results of diagnosis based on consensus clinical impression were compared to histopathology.
4.2.3 Image Analysis

White light reflectance images of each site were first examined; if a clinically apparent lesion was present, a region of interest (ROI) corresponding to the lesion was defined. If the area was clinically normal, a representative ROI was selected from the white light reflectance image. The same ROI was identified in each color fluorescence image of that site, and quantitative image features were calculated for each ROI.

Images were analyzed to yield possible features for use in classification algorithms. The following metrics were generated for ROIs corresponding to lesions and contralateral normal measurements: the average intensity in the red, green and blue (RGB) channels, average values of the ratios of the R/G, R/B and B/G intensities, the average intensity following grayscale conversion, and the standard deviation of the RGB and grayscale intensity values. In addition, for each ROI corresponding to a clinical lesion, we calculated the ratio of the metric for the lesion relative to that measured from the contralateral normal ROI in the same patient. We refer to these metrics as 'normalized ratios'. For measurements from clinically normal sites, normalized ratios were obtained by dividing each ROI in two and calculating the ratio of the metrics from the two resulting regions.

We explored which of these features provided the best separation between non-neoplastic oral mucosa and neoplastic oral mucosa. For calculation of sensitivity and specificity, sites with a diagnosis of ‘Cancer’ or ‘High Risk’ were considered to be neoplastic, while sites with a clinical diagnosis of ‘Normal’ or ‘Low Risk’ were considered to be non-neoplastic. Prior to feature selection, sites with a clinical descriptor of melanosis were excluded from the data set. These sites were easily identifiable from the white light reflectance images. Binary classification algorithms were developed using linear discriminant analysis with a single image feature as input. The same clinical
dataset was used to both develop the algorithm and to assess classification accuracy. For each image feature, diagnostic performance was assessed as the threshold was varied from the minimum to the maximum of its value to generate a receiver operating characteristic (ROC) curve. Classification performance measures, such as the area under the receiver operating curve (AUC), sensitivity and specificity at the Q-point, were calculated for each of the input metrics.

4.3 Results

Images from a total of 351 different anatomic sites were included in the analysis. 261 of these sites were imaged from 76 patients and 90 sites were imaged from 33 normal volunteers. Table 4.1 provides a summary of the number of sites imaged by clinical descriptor and diagnostic category. A total of 222 sites had an initial clinical impression of normal and 129 sites had an initial clinical impression of abnormal. Of the 129 abnormal sites that were later reviewed by expert observers, 8 sites with a clinical description of melanosis and 2 sites with a clinical description of OSF were later assigned a diagnosis of ‘Normal’. Pathologic diagnosis was available for a total of 57 sites imaged; Table 4.2 summarizes the pathologic diagnosis and diagnostic category for these sites.

Figure 4.1 shows typical white light reflectance and fluorescence images of each of the four diagnostic categories. Fluorescence images of sites with a diagnostic category of ‘Normal’ showed homogenous green mucosal fluorescence. Sites with a diagnostic category of ‘Low Risk’ or ‘High Risk’ exhibited a progressive loss of green fluorescence, while sites with a diagnostic category of ‘Cancer’ typically showed both a loss of green fluorescence and an increase in orange-red fluorescence.
Table 4.1. Clinical Descriptors vs. Diagnosis of Measured Oral Sites

<table>
<thead>
<tr>
<th>Clinical Descriptors</th>
<th>Diagnostic Category</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Low Risk</td>
</tr>
<tr>
<td>Normal</td>
<td>222</td>
<td>0</td>
</tr>
<tr>
<td>Melanosis</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>Lichen Planus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oral Submucous Fibrosis</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Leukoplakia</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Leukoplakia/Hyperkeratosis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leukoplakia/Ulcer</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Erythroplakia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ulcer</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Ulcer/Cancer</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cancer</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>232</strong></td>
<td><strong>60</strong></td>
</tr>
</tbody>
</table>
Table 4.2. Pathology vs. Clinical Diagnosis for Measured Oral Sites

<table>
<thead>
<tr>
<th>Pathology Status</th>
<th>Diagnostic Category</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Low Risk</td>
</tr>
<tr>
<td>None</td>
<td>232</td>
<td>53</td>
</tr>
<tr>
<td>Non-neoplastic</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCC</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>232</strong></td>
<td><strong>60</strong></td>
</tr>
</tbody>
</table>
**Figure 4.1.** Images of lesions typical of the four diagnostic categories. White light images are shown on the left and fluorescence images on the right. The top row illustrates 'Normal' buccal mucosa, illustrating homogenous green fluorescence. The second row shows a site with a 'Low Risk' lesion of the buccal mucosa, with loss of fluorescence. The third row shows a site with a 'High Risk' lesion in the buccal mucosa with loss of fluorescence. Bottom row illustrates 'Cancer' of the tongue, illustrating loss of green fluorescence and presence of orange-red fluorescence. Lesions are outlined in the image.

**Figure 4.2.** White light images (left column) and fluorescence images (right column) of typical lesions with OSF (top row) and melanosis (bottom row). Areas with OSF show slightly increased green fluorescence. Melanosis is easily recognizable from the dark pigmentation in the white light image, and is associated with loss of fluorescence.
Table 4.3. Area Under the ROC Curve (AUC) of Top Five Image Features for Binary Classification of Oral Sites

<table>
<thead>
<tr>
<th>Features</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normalized MFI Green channel</td>
<td>0.91</td>
</tr>
<tr>
<td>Normalized MFI Blue channel</td>
<td>0.91</td>
</tr>
<tr>
<td>Normalized MFI Red / Green channel ratio</td>
<td>0.90</td>
</tr>
<tr>
<td>Normalized MFI Grayscale</td>
<td>0.89</td>
</tr>
<tr>
<td>Normalized MFI Red channel</td>
<td>0.85</td>
</tr>
</tbody>
</table>

MFI: Mean Fluorescence Intensity

Figure 4.2 shows white light reflectance and fluorescence images of two potentially confounding lesions found in the study population — oral submucous fibrosis (OSF) and melanosis. In white light reflectance images, OSF sites exhibited a pale-white patchy appearance similar to leukoplakia. However, unlike leukoplakia which is often associated with decreased green fluorescence, no loss of fluorescence was observed in sites with a clinical description of OSF sites. Melanosis sites were easily recognized from the white light images as areas of dark pigmentation and were consistently associated with decreased green fluorescence.

Algorithms based on individual image features were ranked according to the area under the ROC curve (AUC); Table 4.3 lists the AUC for the five algorithms with the best performance relative to expert diagnosis; all were based on normalized features of fluorescence images. The top three performing algorithms were based on the normalized mean fluorescence intensity (MFI) of the blue channel, the normalized MFI of the green channel, and the normalized ratio of red to green MFI. Linear discriminant analysis
involving combinations of image features did not significantly improve classification performance.

Figure 4.3 shows scatter plots of the image features and corresponding ROC curves for classification algorithms which resulted in the best sensitivity and specificity respectively. Figure 4.3a shows the normalized MFI from the blue channel by diagnostic category for all sites. Those sites with a diagnosis of Normal and a clinical descriptor of melanosis or OSF are shown separately. Sites with a diagnosis of ‘Low Risk’, ‘High Risk’ or ‘Cancer’ exhibit decreased normalized blue MFI compared to ‘Normal’ sites. Sites with melanosis show decreased normalized blue MFI, while those with OSF have similar normalized blue MFI to other ‘Normal’ sites. Figure 4.3b shows the ROC curve for the algorithm based on normalized blue MFI, excluding melanosis from the analysis. The operating point indicated on the ROC curve corresponds to a sensitivity of 92% and a specificity of 84%; the threshold values corresponding to this operating point is a normalized blue MFI of 0.86, which is indicated by the solid horizontal line in Fig. 3a.
Figure 4.3. (a) Scatter plot of the normalized blue MFI for all sites measured by diagnostic category. (b) ROC curve for diagnostic algorithm based on normalized blue MFI to differentiate between non-neoplastic sites (Normal, LR and OSF) and neoplastic sites (HR and Cancer). Measurements with melanosis were excluded from algorithm development and testing. The open circle on the ROC curve indicates the Q-point. (c) Scatter plot of the normalized ratio of red to green MFI for all sites measured by diagnostic category. (d) ROC curve for diagnostic algorithm based on normalized ratio of red to green MFI to differentiate between non-neoplastic sites (Normal, LR and OSF) and neoplastic sites (HR and Cancer). Measurements with melanosis were excluded from algorithm development and testing. The open circle on the ROC curve indicates the Q-point.
Figure 4.3c shows a scatter plot of the normalized ratio of red to green MFI by diagnostic category for all sites. Sites with a diagnosis of 'Low Risk', 'High Risk' or 'Cancer' exhibit an increased normalized red to green MFI ratio compared to 'Normal' sites; the normalized red to green MFI ratio increases on average as the severity of the diagnostic category increases from 'Low Risk' to 'Cancer'. Sites with melanosis show an increased normalized red to green MFI ratio, while those with OSF have a similar normalized red to green MFI ratio as other 'Normal' sites. Figure 4.3d shows the ROC curve for the classification algorithm based on the normalized red to green MFI ratio, excluding melanosis from the analysis. The operating point indicated on the ROC curve corresponds to a sensitivity of 90% and a specificity of 87% relative to the gold standard of consensus clinical impression. The threshold value corresponding to this operating point is a normalized red to green MFI ratio of 1.11, and is indicated by the solid horizontal line shown in Fig. 3c.

4.4 Discussion

This pilot study demonstrates that objective analysis of fluorescence images obtained with a low-cost imaging system can classify sites as neoplastic or non-neoplastic with high sensitivity and specificity relative to the gold standard of consensus clinical impression. The performance of this low-cost, objective system compares favorably to results reported for pilot studies of other optical imaging systems. A recent review by De Veld summarizes several clinical studies of optical imaging with qualitative image analysis for detection of oral neoplasia and reports sensitivity ranging from 63% to 100% and specificity from 79% to 96%. More recently, Lane et al. achieved a sensitivity of 98% and specificity of 100% using qualitative assessment of fluorescence images.
acquired with the VELScope to discriminate dysplasia and cancer from normal oral mucosa. Roblyer reported quantitative analysis of fluorescence and reflectance images obtained with a multi-spectral digital microscope to classify oral premalignant lesions and oral cancer with a sensitivity of 94% and specificity of 87%. Using the low-cost optical imaging system described here, we find similar sensitivity and specificity.

The optical characteristics of oral lesions found in this high-risk population are similar to those in other studies. Our finding of decreased fluorescence associated with neoplastic lesions is consistent with reports in the literature. This loss of autofluorescence has been attributed to a decrease in collagen cross-links associated with neoplastic transformation. Our results also indicate a relative increase in red fluorescence for neoplastic sites. Other studies have made similar observations, attributing this increased red fluorescence to porphyrins. In addition, our threshold value for normalized red to green MFI ratio of 1.11 to differentiate neoplastic sites from non-neoplastic tissue is well in agreement with that of 1.09 found by Roblyer at 450-nm excitation.

We characterized the optical properties of two types of potentially confounding lesions, melanosis and OSF, specific to the geographic region. Melanosis is usually benign and not considered to be precancerous. Our results indicate that sites with melanosis exhibit decreased fluorescence, but can easily be recognized by their characteristic appearance in white light reflectance images. The loss of autofluorescence in melanosis is likely due to strong absorption of light by black pigmentation in the superficial epithelium. In contrast to melanosis, the malignant transformation rate of OSF has been estimated to be between 3% and 19%. Most of the OSF sites measured in this
study were graded as 'Low Risk'. We found that the OSF sites imaged here did not exhibit loss of fluorescence. Histologically, OSF is characterized by juxtaepithelial fibrosis, along with atrophy or hyperplasia of the overlying epithelium, keratinizing metaplasia, and accumulation of hyalinized collagen beneath the basement membrane. All of these constituents are strong sources of autofluorescence and may contribute to autofluorescence observed in our images of OSF sites.

While the sensitivity and specificity reported here are encouraging, there are a number of limitations of this study. First, the same dataset was used both to develop classification algorithms and to assess their performance; in this situation, potential over-training can inflate estimates of sensitivity and specificity. Results must be verified in an independent validation set. Second, the gold standard used to assess algorithm performance was consensus clinical impression; due to resource limitations, histopathologic diagnosis was not available from all sites. Table 2 provides a comparison of consensus clinical impression to histologic diagnosis of the 57 sites for which histology was available. Using histology as a gold standard to evaluate the ability of consensus clinical impression to discriminate neoplastic and non-neoplastic sites, results in a sensitivity of 96% (44/46); too few non-neoplastic sites were biopsied to estimate specificity of clinical impression in this study. Finally, a large number of 'Low Risk' sites were misclassified as neoplastic by the optical algorithms presented here. It is interesting to note that 'Low Risk' sites with consensus among all three expert observers were more likely to be classified by the optical algorithms as non-neoplastic (6/10 = 60%) than were 'Low Risk' sites where only two of the expert observers agreed (13/30 = 43%). Additional studies with histologic endpoints for all sites are required to assess the
ability of quantitative optical image analysis to aid in the evaluation of low risk oral lesions.

4.5 Conclusion

The clinical study presented here demonstrates the ability to identify neoplastic and non-neoplastic oral tissue in vivo objectively using the portable, low-cost imaging system. Although further work is needed to address the limitations in this study, results from this pilot study suggest that this simple imaging device can potentially improve oral screening efforts in low-resource settings where clinical expertise and resources are often limited.
5.1 INTRODUCTION

In the past decade, optical technologies have emerged as promising tools for the diagnosis of various epithelial cancers including esophagus, oral, and cervical pre-cancer. The need for noninvasively screening of large populations for these cancers has motivated the advancement of optical imaging technologies such as optical coherence tomography (OCT) and confocal microscopy. Due to their ability to provide diagnostic information at sub-cellular spatial resolution in real-time, optical imaging tools have the potential to better direct or replace routine biopsies in cancer screening and early detection. Moreover, the sensitivity and specificity of these optical methods has the potential to surpass that of conventional histopathology. However, more emphasis is needed to optimize these tools for use in vivo imaging as well as to examine other optical imaging alternatives which produce the same diagnostic ability but at a lower cost. This is crucial since large percentages of the population most at risk to develop oral, cervical and other cancers are found in developing countries where cost is a major deterrent to routine screening. Thus, alternative methods to high-cost screening, which can perform real-time diagnosis, can have a major impact on reducing mortality rates.

Confocal scanning optical microscopy has emerged as a technique which exhibits several advantages over conventional optical microscopy. The important feature of confocal microscopy is the fact that out-of-focus blur is absent in confocal images, yielding the capability for non-invasive optical sectioning of both in vivo and in vitro specimens. This leads to the possibility of generating (3D) images of thick transparent objects such as biological cells and tissues. The simple and inexpensive alternative to confocal microscopy is the structured-illumination technique proposed by Neil, et al.\textsuperscript{77} This method uses the principle that all but the zero (a.k.a. DC) spatial frequencies are attenuated with defocus. This observation provides the basis to obtain optically sectioned images from a conventional widefield microscope. A modified illumination system of the microscope projects a single spatial frequency grid pattern onto the object. The microscope then images faithfully only that portion of the object where the grid pattern is in focus. The structured-illumination technique requires acquisition of at least three images to remove illumination structure and reconstruct an image of the layer. The technique has been already applied to small pollen grains in reflectance\textsuperscript{77} mode and cell monolayers in fluorescence mode.\textsuperscript{78} Recently, the technique was implemented commercially by ZEISS\textsuperscript{100} in the ApoTome and Thales Optem in OptiGrid\textsuperscript{101} heads for inverted microscopes and fluorescent applications. Additionally the system was commercialized for industrial applications (MEMS testing) by Thales Optem.\textsuperscript{101}

Recently, confocal microscopy has shown promise to image the morphologic features of precancerous lesions in epithelial tissue.\textsuperscript{13,58} The simplicity of the structured illumination technique provides a convenient alternative to obtain this useful diagnostic information. There is however no example of successful reflectance and fluorescence
structured illumination imaging of dense tissue samples. The difficulty, especially in reflectance imaging, arises from the very strong out-of-focus contributions compared to the optical-section signal. The useful in-focus information is limited to even as low as two gray levels of the camera, and standard algorithms are not capable of effectively reconstructing images of the in-focus section under these conditions. Furthermore, reflectance imaging of tissue is generally sensitive only to non-specific differences in refractive index, and provides rather limited information about the range of molecular changes associated with patho-physiology. Therefore, to overcome these problems two actions were undertaken: (1) the sine approximation algorithm was applied for image reconstruction and (2) targeted contrast agents were used to provide a molecular specific source of increased signal from cells overexpressing cancer-related biomarkers.

Sine approximation algorithm differs from conventional structured illumination reconstruction in that more images per sample layer are acquired to account for non-optimized parameters in structured illumination imaging. These non-optimized parameters can include: (a) a systematic error in the stepping size of grid pattern, (b) random additive noise, (c) contribution of out-of-focus scattered light and (d) limited dynamic range of the detector. A detail discussion of this algorithm addressing each of these parameters can be found in Ref 102. To enhance the contrast for imaging of precancerous specimens, contrast agents that are molecular specific to cancer biomarkers were utilized. Antibodies against the epidermal growth factor receptor (EGFR), a receptor commonly overexpressed in cancerous and precancerous tissue, was used to target the contrast agents. After integrating these two approaches, high resolution images were obtained from biological specimens that were representative of normal and
precancerous tissue. For this, multi-layer epithelial cell phantoms were created with cells expressing levels of EGFR typical for normal and neoplastic tissue. These phantoms were used to demonstrate the optical sectioning ability of structured illumination in optically dense tissue samples and to image morphologic features of precancerous lesions.

Figure 5.1. Layout of the typical structured illumination microscope system. Adapted from ref. 102

5.2 MATERIALS AND METHODS

5.2.1 Structured Illumination Setup

In order to implement structured illumination technique for imaging precancer, a Zeiss Axiovert 100M microscope was modified such that grid patterns can be projected on the focal plane of the microscope. A schematic of the modified microscope is shown in Fig. 5.1. The grid structure was placed at the conjugate focal plane in the back of the
microscope. The rectangular grid structure with 10 lines/mm or 100 μm period (Edmund Optics) was precisely driven by an actuator (SMAC-10) which had a stepping size of 0.5μm. The actuator could be programmed such that it would have all movement positions pre-recorded in its memory and would precisely move to those locations as commanded. An Olympus MD Plan 20x and a Zeiss 63x water immersion objective lens with a N.A. of 0.50 and 0.95 respectively were used for the experiments. A halogen lamp (100 watts) was used as an illumination source. Light from the lamp was collimated and redirected onto back of the objective lens to the sample with a 30/70 beam splitter (Chroma Tech. Corp.). The reflected light from sample passed through the beam splitter and was projected to the eye-piece and camera. All images were acquired using a 12-bit monochrome CCD camera (Hamamatsu Orca-1) mounted on top of the microscope. Both the camera and the actuator were controlled in AxioVision software provided by Carl Zeiss Inc. In order to automate the image acquisition procedure, a program was written in Visual Basic software that could move the actuator driven grid pattern and acquire image from the camera in a synchronizing fashion.

In order to reconstruct optically sectioned image, the sinus approximation algorithm was used. The sine approximation algorithm applied here in the context of structured-illumination has been previously used to monitor vibrations\textsuperscript{103-105} and to measure shape\textsuperscript{100}; however, in the context of image processing, this is a new approach. The algorithm is very robust and, most importantly, it works correctly for significantly smaller dynamic range than is required by conventional structured illumination imaging techniques. In order to evaluate the optical sectioning ability of the structured
illumination system, 15µm fluorescence beads were used as positive standards. Fig. 5.2 shows fluorescence beads and the optical sectioned image.

![Fluorescence images of 15 µm bead](image)

**Figure 5.2.** Fluorescence images of 15 µm bead: widefield image of beads with both in-focus and out-of-focus beads (left), optically sectioned image of panel a with only the in-focus bead obtained in structured illumination (right).

### 5.2.2 Tissue Phantom Preparation

To demonstrate the structured-illumination technique in dense biological tissue, tissue phantoms containing multiple layers of epithelial cells\textsuperscript{72} were prepared. These phantoms have optical, morphologic and molecular properties representative of normal and precancerous squamous epithelial tissue. The tissue phantoms were prepared using SiHa cervical epithelial cancer cells and MDA-MB-435S breast epithelial cancer cells. The SiHa cells overexpress EGFR and provide a good model of neoplastic epithelium, while the MDA-MB-435S breast epithelial cells do not express EGFR, and in this regard, provide a good model of normal tissue. Phantoms of each epithelial cell line were prepared by resuspending cultured cells in a volume of buffered collagen type I to obtain
a cell density of 108 cells/ml. Collagen-cell suspensions were plated into 6.5 mm diameter transwells and allowed to gel at 37°C. The prepared tissue phantoms were allowed to grow in DMEM plus 5% FBS for 24 hours so that they formed a highly dense structure consisting of multiple layers of epithelial cells. The phantoms were determined to have a thickness between 400 and 600 μm.

In order to enhance the reflectance and fluorescence signal from cells, phantoms were labeled with molecular-specific contrast agents. For reflectance imaging with structured illumination, the contrast agent consisted of gold nanoparticles conjugated to anti-EGFR monoclonal antibodies to provide molecular-specificity. The gold nanoparticles were 20 nm in diameter with a peak scattering coefficient between 630 and 680 nm. The monoclonal antibodies are molecular specific to EGFR, which is a transmembrane Mr 170,000 glycoprotein that is overexpressed in epithelial precancers. Both colloidal gold and EGFR antibodies have been used clinically previously. A solution of the anti-EGFR gold contrast agent was applied to the top surface of the prepared phantom for 10 minutes; phantoms were then rinsed with phosphate buffered saline (PBS) and imaged. Labeled MDA-MB-435S phantoms which do not express EGFR were expected to give very little reflective signal compared to that observed with labeled SiHa phantoms. Labeled SiHa phantoms which overexpress EGFR were expected to show clear labeling of cell membranes. As a control experiment to test for non-specific binding, a separate set of phantoms were also labeled with 20 nm diameter gold nanoparticles conjugated to non-specific IgG antibody and imaged; very little reflectance signal was expected under these conditions.
For fluorescence imaging, phantoms were first topically labeled with biotinylated anti-EGFR or non-specific IgG antibody followed by labeling of 655 nm streptavidin quantum dots (10–15 nm diameter) commercially available from QDot Corporation. The standard protocol provided by the manufacturer was followed in the quantum dot labeling procedure. A detail labeling step procedure for this can be found in Chapter 6.

5.2.3 Image Acquisition

Imaging experiments were carried out using a Zeiss 63x magnification water-immersion objective lens with a NA of 0.95. The imaging configuration was diffraction limited and the transverse system resolution was approximately 0.4 μm. The maximum field of view of the imaging system depended on the magnification and parameters of CCD camera (Hamamatsu ORCA C4742-95) and was approximately 143 μm x 108 μm. However for the purpose of this study a region of 512 x 512 pixels was selected which corresponded to field of view of 54 μm x 54 μm. In order to reconstruct optical sectioned image with sinus approximation algorithm, a maximum of 64 images per layer were acquired. For one sample layer, a total acquisition time of 5 to 20 seconds was required.

5.3 Results

Figure 5.3 presents imaging results for a phantom made of SiHa cervical cancer cells labeled with the anti-EGFR gold contrast agent. Figures 5.3(a), 5.3(b), and 5.3(c) show a conventional widefield reflectance microscope image, a structured-illumination raw image, and a reconstructed optical-section image, respectively. The reflectance from the colloidal gold particles can be appreciated at the cytoplasmic membrane of the SiHa cells which overexpress the EGF receptor. This characteristic “honeycomb” pattern is consistent with previous results reported in literature for this contrast agent using
conventional confocal microscopy. The result in Fig. 5.3(c) represents a layer 15–20 μm below the phantom surface.

Figure 5.3. Images of phantoms containing SiHa cervical cancer cells labeled with anti-EGFR gold conjugates. The field of view is 54 × 54 μm². The approximate depth of the imaged optical section is 15-20 μm below the phantom surface. Part (a) shows an inverted widefield reflectancemicroscope image. Part (b) shows a structured-illumination raw image. Part (c) shows a reconstructed optical-section image.

Figure 5.4 shows imaging results for the MDA-MD-435S phantom labeled with the anti-EGFR gold contrast agent. Figure 5.4(a) and 5.4(b) show a structured-illumination raw image and a reconstructed optical-section image, respectively. The image shown in Fig. 5.4(b) represents an optical section approximately 20 μm below the surface of the phantom. The image in Fig. 5.4(b) is one of 25 optical sections scanned with 2 μm axial increments through the tissue phantom. The resulting optically sectioned images of the 435 cells labeled with the anti-EGFR gold are very similar to the control images obtained with both the 435 cells and the SiHa cells labeled with the non-specific control antibody; the SiHa cells labeled with the specific antibody (Fig. 5.3) show much higher intensity as expected. The images in Fig. 5.4 (EGFR-negative cells labeled with the EGFR-specific contrast agent) provide an indication of the ability of structured illumination to image the native reflectance of tissue. In this case, we find that the image
detail present in both structured illumination and confocal images (data not shown) compares favorably, as does the penetration depth.

Figure 5.4. Images of phantom containing MDA-MB-435S cells labeled with anti-EGFR gold conjugates. The field of view is 54 μm × 54 μm. The approximate depth of the optical section is 20 μm beneath the surface of the phantom. Part (a) shows a structured illumination raw image. Part (b) shows a reconstructed optical-section image.

In order to compare the structured-illumination results with a "gold standard" imaging system, confocal microscope images of the SiHa tissue phantom stained with the anti-EGFR gold colloid were collected. The confocal microscope (Leica TCS-4D) was used in reflectance mode at 647 nm illumination with a 63x, NA = 0.95 water immersion objective. Figure 5.5 presents a confocal-microscope image taken at approximately 15 μm below the surface of the phantom. There is a very good match in terms of contrast and detail between the images presented in Fig. 5.3(c) (structured illumination) and Fig. 5.5 (confocal microscopy). It is worth noting that the structured-illumination microscope and the confocal microscope could image the same sample to a maximum depth of 50 μm. We found that this maximum depth was limited by the labeling technique rather than the performance of each instrument and its imaging modality.
Figure 5.5. Reflectance confocal-microscope image of the phantom containing SiHa cells labeled with anti-EGFR gold conjugates. The field of view measures approximately 60 μm × 60 μm. This field of view represents a segment of the full field of view of the confocal microscope chosen in size to match results obtained with structured illumination.

Figure 5.6. Fluorescence image of SiHa cervical cancer cell phantoms optically sectioned with structured illumination. (a) Anti-EGFR targeted with quantum dot phantom representing cancerous tissue, (b) non-specific IgG targeted with quantum dot phantom.
Fig. 5.6 shows an optical sectioned image of quantum dot labeled SiHa cervical cell phantoms in fluorescence mode. As in Fig. 5.3 (c) (reflectance structured illumination) and Fig. 5.4 (reflectance confocal microscope), honeycomb pattern can also be seen with this quantum dot labeled phantom.

5.4 QUANTITATIVE IMAGE ANALYSIS

The reconstructed reflectance structured illumination images were analyzed quantitatively to determine whether image intensity parameters could be used to discriminate images of the phantoms representative of normal and neoplastic tissue. The mean signal intensity in the vicinity of the cell membrane were compared, since this is the area that is labeled with the anti-EGFR gold colloid. The mean reflectance intensity at the vicinity of the cell membrane was found for each of approximately five of the SiHa and 435S cell phantoms individually at a similar sample depth. Images from both phantoms were first multiplied by a single constant factor to enhance their brightness. The NIH freeware program ImageJ was then used to hand-segment the area of the cell membrane and calculate the mean reflectance value within the selected region. The average intensity of the cell reflectance signal was 62.5 gray-scale values for SiHa phantom $M_{neo} = 62.5$, substantially greater than the average 28.5 gray-scale reflectance signal for the MDA-MD-435S cell phantoms ($M_{norm} = 28.5$). The corresponding standard deviations were $\sigma_{neo} = 4.9$ and $\sigma_{norm} = 3.5$, respectively. The difference in the average membrane intensities is greater than the sum of the associated standard deviations, i.e.

$$|M_{neo} - M_{norm}| > (\sigma_{neo} - \sigma_{norm}) : |62.5 - 28.5| = 34.0 > (4.9 + 3.5) = 8.4.$$
5.5 CONCLUSION

In summary, the feasibility of the sine approximation algorithm applied to structured-illumination imaging was demonstrated. Additionally structured-illumination imaging in reflectance and fluorescence mode of optically dense tissue phantoms were successfully performed. The results compare very favorably with "gold standard" confocal microscope images of the same phantom. The phantoms representative of the optical and molecular properties of neoplastic and normal tissue labeled with a simple optically active, molecular-specific contrast agent can be distinguished quantitatively using optical-section image data that have been collected and an image-analysis metric that is sensitive to the difference in labeling based on molecular biomarkers of precancer and cancer.
Chapter 6

Imaging performance of a miniature integrated microendoscope

6.1 OVERVIEW

This chapter presents imaging performance of a miniature microendoscope developed by Descour et al.\textsuperscript{108} for early detection of oral cancer. The instrument is designed to image in fluorescence or reflectance, in widefield, or with optical sectioning using structured illumination.\textsuperscript{77} The performance of the integrated miniature microscope was evaluated by imaging fixed biological samples acquired by the microendoscope to demonstrate its ability to image the cellular structure of tissue. This multi-modal miniature microscope is referred to as the 4M device. A rendering of the complete 4M device conceptual design is shown in Figure 6.1.

Descour et al. presented the concept, design, and fabrication technology used in the 4M device, including descriptions of the mounting method and conceptual system design,\textsuperscript{108-111} printed lens fabrication,\textsuperscript{112} optical design for stray light reduction,\textsuperscript{113} detector design,\textsuperscript{114} and a method of optical sectioning.\textsuperscript{102} The emphasis of this research work was the demonstration of imaging cultured cells and prepared tissue \textit{in vitro} using the 4M system.

Figure 6.1. Rendered design of the 4M device that illustrates optical components mounted on the MOT substrate. In the future completed device (not shown), the image sensor is to be mounted on a PCB and attached to the 4M device walls. Adapted from ref 116.

The 4M device has a numerical aperture (NA) of 0.4 and a magnification of 4x with a 250 μm-diameter field of view (FOV) and a working distance of 300 μm. The device uses a 650-nm peak wavelength for reflectance imaging and 450-nm excitation with 655-nm emission for fluorescence imaging. The source used for each mode are high brightness LED of the appropriate peak wavelength, which is coupled to the system by a power delivery fiber.

6.2 Imaging of Biological Specimens

The long-term goal of the research work is to utilize the 4M device to detect precancerous lesions in squamous epithelial tissues especially in the oral cavity and cervix. To image the morphological and biochemical changes associated with precancer in these sites, multiple imaging modalities may be required. Thus, two 4M devices with different modalities were considered for imaging relevant biological specimens: one
setup with a fluorescence imaging capability, and the other with a reflectance imaging capability. The differences between the two systems were the illumination sources and the beamsplitter coatings, as described earlier. To evaluate the performance of the 4M device in each of the modalities, exogenous contrast agents were used to label the biological specimens. The contrast agents provided two advantages: an increased SNR of the samples compared to their native contrast, and molecular imaging of specific biomarkers of interest, specifically the epidermal growth factor receptor (EGFR) in human oral precancerous cells.

For fluorescence imaging with the 4M device, samples were labeled with quantum dots. Quantum dots are highly stable semiconductor nanocrystals with a high quantum yield, which results in a much higher signal and SNR than typical autofluorescence signals from tissue. Moreover, their emission wavelengths are tunable with their size, allowing one to image in the near-infraRed (NIR) region of the spectrum where the penetration depth of light in tissue is higher. For this study, CdSe quantum dots (QD655, Invitrogen) were chosen, which have a broad excitation range from 300 to 500 nm and a narrow emission peak at 655 nm. For reflectance imaging with the 4M device, the specimens were labeled with 40 to 55-nm gold nanoparticles. Recently, gold nanoparticles were shown to be potential contrast agents for in vitro molecular imaging of precancerous cells by Sokolov et al.\textsuperscript{72} and El-Sayed et al.\textsuperscript{115} The particles have the ability to resonantly scatter light in the visible and NIR wavelengths depending on their size and shape. The protocol provided in Ref. 72 was followed to synthesize the 40 to 55-nm gold particles, which had a peak scattering coefficient wavelength between 630 and 680 nm.
Since the goal in this work was to demonstrate the high resolution imaging ability of the 4M device optical system in relevant biological samples, biological specimens that closely approximated those organ sites with a squamous epithelium where the 4M system is intended to be used were chosen—for example, the oral cavity and cervix. Due to the structural and biomolecular similarity between the oral cavity and cervix, only oral epithelial cells and tissue were chosen for the experiments. As mentioned earlier, contrast agents were used for molecular imaging of EGFR molecules, which are transmembrane glycoproteins that are over-expressed in both oral and cervical premalignant cells and tissue.\textsuperscript{106-107} Initial experiments were carried out on 3-D tissue phantoms made from 1483 human oral carcinoma cells. Phantoms were prepared by re-suspending approximately 10 million cells in a volume of buffered collagen type I according to a procedure developed by Sokolov et al.\textsuperscript{72,102} Collagen-cell suspensions were plated into 6.5-mm-diameter transwells and allowed to grow for 24 hours to allow the cells to develop a 3-D network within the collagen matrix. Prior to imaging, the phantoms were labeled with appropriate contrast agents.

To label the phantoms with quantum dots, an indirect two step labeling procedure was followed. Prior to primary labeling, the phantoms were incubated in a 1% bovine serum albumin (BSA) solution for 15 minutes to block any nonspecific labeling. The phantoms were then incubated in a 500 \( \mu \)L solution of biotinylated anti-EGFR (clone 111.6, LabVision). After 1 hour of primary labeling at 24°C, excess antibodies were removed from the phantoms by washing them in phosphate buffered saline (PBS) two times.
A 500 μL solution of 10-nM streptavidin-coated quantum dots was prepared in a 1xPBS for secondary labeling. After 30 minutes of secondary incubation at 24°C, the phantoms were again washed in PBS to remove unbound quantum dots. Appropriate control samples, including nonspecific biotinylated anti-human goat IgG (BA-3000, Vector) and secondary only labeling, were also prepared. To label phantoms with gold nanoparticles, antibody-gold conjugates were first prepared according to the protocol presented in Ref. 72. The procedure involved preparing a mixture of colloidal gold particles and anti-EGFR monoclonal antibodies at 0.10 mg/mL (clone 29.1, Sigma) in a 20-mM HEPES buffer with a subsequent addition of polyethylene glycol (PEG) and centrifuging the mixture at 5000 rpm for 2 hours to collect the bound conjugates. The phantoms were then incubated in a 500 μL solution of the prepared conjugates for 1 hour at 24 °C in the presence of 10% dimethyl sulfoxide (DMSO) and washed twice in PBS to remove the unbound conjugates. DMSO was used as a permeation enhancer for the contrast agents. Control phantoms were similarly incubated with a nonspecific mouse IgG antibody-gold conjugated solution and washed in PBS. All phantoms were fixed in 10% formalin after labeling to preserve their natural structural integrity prior to imaging with the 4M devices.

The imaging setup for both of the 4M devices were conducted on an optical relay system with a color CCD camera (EC01380, Prosilica) as mentioned earlier. In fluorescence mode, the gain setting of the CCD was increased to optimize the signal-to-background ratio. The CCD was color-balanced prior to the imaging experiment. All imaging experiments were carried out in a PBS immersion for index matching by
bringing the 4M device lens into contact with the wet sample and then focusing back to
the tissue plane of interest. The surface tension maintained the immersion of the 4M
device to distances well beyond the working distance of the 4M device. The specimens
were transferred to microscope slides and mounted on a micrometer for precise focusing
of the sample on the 4M device. To compare the performance of the 4M devices, all
samples were subsequently imaged with a Zeiss AxioImager Z1 microscope. A 20x dry
objective lens with a NA of 0.6 was used for imaging, and images were acquired with a
color CCD camera (AxioCam Mrc5, Zeiss). A quantum dot 655-nm filter set (Chroma
Technology, U.S.) was used to image the fluorescence samples.

6.3 RESULTS

Figure 6.2 shows widefield fluorescence images of 1483 oral carcinoma cells in
phantom acquired with the Zeiss AxioImager Z1 microscope and with the 4M device.
The last image in the row is the IgG control specimen. The results show clear labeling of
the targeted biomarkers around the cell membrane in the EGFR-positive sample, but no
labeling in the control sample.

Figure 6.3 shows widefield reflectance images of the phantoms labeled with gold
nanoparticles. The widefield images show the morphological pattern characteristic of
single backscattering; however, the background associated with multiple light scattering
Figure 6.2. Widefield images of cells labeled with fluorescent quantum dots imaged with the 4M device (center) and with a Zeiss Axiovert 100M for comparison (left). Cell membranes are clearly visible in each image. A control sample with nonspecific IgG labeling is shown on the right. The scale bar is 25 μm.

Figure 6.3. Widefield image of cells imaged in reflectance labeled with gold nanoparticles. The image taken with the 4M device (right) shows the significance of the internal lens surface reflections. The bright region on the right of the image is due to a single surface reflection from an AR-coated lens. A similar image taken with a Zeiss Axiovert 100M is shown (left) for comparison. Black circular masks limit the FOV to 250 μm for each image.
from cell layers underneath reduces the image contrast. The 4M image is intermediate between the two—the single backscattering morphology is clearly visible in the center of the field, but the multiple scattering again reduces the image contrast somewhat. These results clearly demonstrate the ability to image backscattered light from cells in the current 4M device.

To demonstrate the imaging feasibility of the 4M device with more complex biological specimens, porcine epithelial tissue were chosen because it is readily accessible and its morphological features are somewhat similar to those of human epithelial tissue. For sample preparation, fresh tissues were obtained from a local abattoir, and 6-mm punch biopsies were taken from the buccal mucosa of the porcine oral cavity. The tissues were then fixed in 10% buffered formaldehyde for 24 hours to preserve their structural integrity for a longer period. The tissues were sliced transversely in 200 to 300 μm thickness using a Krumdieck tissue sheer (Munford, Alabama). Tissue slices were subsequently labeled with contrast agents. For fluorescence imaging, the tissues were labeled with streptavidin-coated 655-nm quantum dots using the two steps indirect procedure described earlier. For positive labeling, biotinylated anti-swine IgG (BA-9020, Vector Lab) antibodies were used (0.15 mg/mL in 1x PBS). Samples were also prepared that included primary labeling with anti-mouse IgG (Labvision) and secondary-only labeling for control. No attempt was made to label the porcine tissues with gold nanoparticles due to the 4M device’s lack of contrast in reflectance mode as experienced with the phantoms. Figure 6.4 shows widefield images of the porcine epithelial tissue taken with the Zeiss AxioImager Z1 microscope and with the 4M device. Although the
signal was weak in the 4M image due to a limitation in the excitation power, squamous epithelium cells can still be appreciated from the image.

Figure 6.4. Widefield fluorescent images of porcine epithelial tissue labeled with quantum dots taken with a Zeiss AxioImager Z1 (left) and with the 4M device (center). Squamous epithelial cells can be appreciated in both images. A control image is shown on the left. The scale bar is 25 μm.

6.4 CONCLUSIONS

The results presented here demonstrate the ability to image fixed biological samples using the miniaturized microscope. Both reflectance and fluorescence imaging modalities can be used to acquire high-quality images of cells and epithelial tissues in vitro. Although further work is needed to use the device in vivo in a clinical environment, the results suggest that the 4M device can be used with targeted contrast agents to image the molecular features of cancer and its precursors. Furthermore, its application can potentially be extended to other areas of disease diagnosis where widefield microscopy is commonly used.
Chapter 7
Conclusions

7.1 Summary of Results

The main objective of this research was to develop optical imaging systems that are low-cost, portable and can aid in screening and detection of oral cancer. Recent cancer facts and figures indicate that over two-thirds of oral cancer cases and three-quarters of deaths due to the disease occur in developing countries where resources are often limited. No satisfactory mechanism exists currently to screen and detect early neoplastic changes of the oral cavity in the general population. Optical imaging technologies have the potential to aid in the identification of oral cancer and its precursors. Translating these technologies to developing countries and low-resource settings where the vast majority of oral cancers occur, thus, can be an important contribution to global efforts to reduce oral cancer mortality and morbidity.

This dissertation presented two imaging systems that are low-cost, portable and can aid in screening and detection of oral cancer in resource limited settings. The first system is a low-resolution wide-field imaging system designed to screen surface area of oral tissue at risk to detect abnormal sites with high sensitivity. The second system is a high-resolution microscopic system designed to detect molecular changes in tissue to achieve high diagnostic specificity. In addition to developing the above two imaging platforms, the imaging performance of a miniature microscope developed by Descour et al. was evaluated as a part of this dissertation work.
The third chapter of this dissertation describes the design and construction of a low-resolution wide-field imaging system that is low-cost, portable and has multi-modal imaging capabilities in-vivo. Simple modifications to a surgical headlight system were made that enables direct visualization and digital image acquisition from oral tissue in multiple imaging modes including fluorescence, white-light reflectance, and orthogonal polarization reflectance. The multi-modal imaging system uses light emitting diodes (LEDs) to illuminate the oral mucosa. For fluorescence imaging, the system has a blue LED with an excitation peak at 455 nm wavelength; for white-light reflectance imaging, it has a white LED with an illumination range of 400 to 700 nm. Images can either be observed visually or captured digitally through a set of optical filters using an integrated, miniature charge coupled device (CCD) camera. The system is connected to a laptop via a firewire interface to record and store the images. The portable system weighs only 3 pounds and can be powered by a lithium-ion battery. Overall, the design of this system makes it low maintenance, affordable and adaptable to adverse conditions in developing countries and remote settings. The chapter ends with presentation of a brief set of clinical images obtained from patients in-vivo. The results demonstrate that it is an attractive technology to explore for oral cancer screening in low-resource environments where clinical expertise is often unavailable.

The fourth chapter of this dissertation presents evaluation of the simple, low-cost wide-field imaging system described in Chapter 3 in a resource limited setting where oral cancer is prevalent. A clinical study was conducted at Tata Memorial Hospital (TMH) in Mumbai, India using this system. Reflectance images with white light illumination and fluorescence images were obtained from 261 sites in the oral cavity from 76 patients and
90 sites in the oral cavity from 33 normal volunteers. Quantitative image features were used to develop classification algorithms to identify neoplastic tissue, using clinical diagnosis of expert observers as the gold standard. Using the ratio of red to green autofluorescence, the algorithm identified tissues judged to be cancer or clinically suspicious for neoplasia with a sensitivity of 90% and a specificity of 87%. The study found optical characteristics of oral lesions in this high-risk population to be similar to those in other similar studies reported in the literature. The study also investigated the optical properties of two potentially confounding lesions that is population specific to south Asia. Although there were several limitations to this study, results from the pilot study suggest that the performance of the simple, objective low-cost system has potential to improve oral screening efforts, especially in low-resource settings.

The fifth chapter describes modification of a conventional wide-field microscope to obtain high-resolution imaging as an alternative approach to complex and expensive confocal microscopy. Novel, optically active contrast agents (i.e. gold nanoparticles and quantum dots) were utilized to label disease-related biomarkers and increase the signal to signal-to-noise ratio (SNR) in structured illumination microscopy of biological tissue. Reflectance and fluorescence imaging results for multi-layer epithelial cell phantoms with optical properties characteristic of normal and cancerous tissue labeled with the contrast agents were presented. Structured illumination images reconstructed with the sine approximation algorithm compared favorably to those obtained with a standard confocal microscope. The results demonstrates that the concept of integrating structured illumination with optically active contrast agents can be a useful tool for simple and small imaging platforms for high-resolution molecular-specific imaging of precancer.
The sixth chapter describes imaging performance of a multi-modal miniature microscope (4M device) developed by Descour et al. Imaging performance was evaluated in fluorescence and reflectance mode using thin layers of fixed cells and excised porcine epithelial tissue from the oral cavity. Imaging results suggest that the 4M is capable of imaging biological specimens and has potential for in vivo applications.

7.2 Future Directions

The pilot study conducted for clinical evaluation of the PS2 system demonstrates that the system has the potential for screening and early detection of oral cancer with a high sensitivity. However, a large number of oral sites that were graded as 'Low Risk' by the expert observers in the study were misclassified as neoplastic tissue, resulting in poorer specificity than desired. This can lead to a large number of false positives in the general screening population. One approach to address this limitation would be to utilize high-resolution optical imaging system that has higher specificity to eliminate the false positives from the wide-field imaging system. Various high-resolution optical imaging methods including structured-illumination described in the second part of this dissertation has been proposed for early detection of precancer and cancer. Of particular interest is the low-cost fiber microendoscopy system developed by Muldoon. The author has demonstrated that the microendoscopy system could qualitatively and quantitatively differentiate between normal and cancerous tissue based on sub-cellular image features.

Future studies that can combine the two imaging systems of wide-field imaging and high-resolution imaging to achieve high sensitivity and specificity would be an important contribution towards assessment of optical imaging as screening tool for oral cancer. Due to limited field-of-view in high-resolution imaging systems, the fiber
microendoscopy does not allow screening of surface area at risk. The wide-field imaging capability of the PS2 system can be used for identification of oral tissue that is at risk while the fiber endoscopy can be used for detailed assessment of the suspicious areas to detect a variety of morphologic, architectural, biochemical and molecular features of early neoplasia. Further, quantitative analysis of image features from the two systems can be combined to determine whether the two imaging systems together will yield classification schemes with higher sensitivity and specificity. Finally, because of the low cost and portability of these two optical systems, the study can be easily conducted in a resource-limited region with high-risk populations for oral cancer.

7.3 Conclusions

The two imaging systems described in this dissertation can potentially be useful tools for screening and early detection of oral cancer resource limited settings. In particular, the low-cost, portable imaging system can become a valuable aid for health care professionals in resource limited settings where clinical expertise is unavailable for identification of cancer and precancerous lesions.
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