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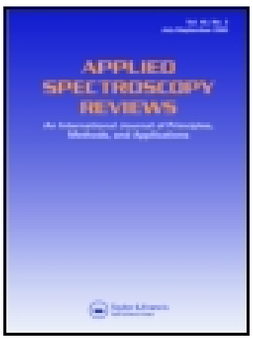

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Applications of raman spectroscopy in dentistry part II: Soft tissue analysis

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Abstract

Raman spectroscopy is rapidly moving from an experimental technique for the analysis of biological molecules to a tool for the real-time clinical diagnosis and in situ evaluation of the oral tissue in medical and dental research. The purpose of this study is to identify various applications

of Raman spectroscopy, to evaluate the contemporary status and to explore future directions in the field of dentistry. Several in-depth applications are presented to illustrate Raman spectroscopy in early diagnosis of soft tissue abnormalities. Raman spectroscopy allows to analyze histological and biochemical composition of biological tissues. The technique not only demonstrates its role in the disclosure of dysplasia and malignancy but also in performing guided biopsies, diagnosing sialoliths, and assessment of surgical margins. Raman spectroscopy is used to identify the molecular structures and its components to give substantial information about the chemical structure properties of these molecules. In this paper, we acquaint the utilization of Raman spectroscopy in analyzing the soft tissues in relation to dentistry.

Keywords

Raman spectroscopy, Dental tissue, Oral soft tissue analysis

Introduction

Soft tissue is an accumulation of cells and extracellular matrix. Cells of any tissue carry out specific function within the organ. On this basis, all the organs, structures and other elements of a multicellular organism can be assigned to a specific tissue in the body. Study of a tissue is termed as histology and its pathology is defined as histopathology, which is regarded as a gold standard to study properties of natural tissue. In last few decades, histopathology has improved significantly with the invent of digital pathology or whole slide imaging, which involves digital image capturing at different wavelengths and later computer analysis. Later introduced electron microscopy and fluorescence microscopy significantly elevated our knowledge in terms of structure and function of tissue. However, all these techniques need considerable specimen preparation for analysis. Furthermore, there is limited approachability of the label to the target, insufficient binding specificity and a poor stability (1, 2).

Quest for new techniques to identify structural and functional properties has been the focus of attention and as a result a number of new analytical techniques have been added to the armory of both clinical and non-clinical researchers. Raman and Fourier Transform Infrared (FTIR) spectroscopic techniques have been used to study chemical structural properties of cells, fresh and fixed tissues, normal and diseased tissues, and drug interactions with the tissue allowing to identify chemical pathway of these interactions. Both these techniques are complimentary to each other, sensitive and rapid (3-12). Raman spectroscopy is based on the Raman scattering or Raman effect i.e., inelastic dissipating (approximately 1 in 10^6 to 1 in 10^8 photons) of monochromatic visible (400-700nm) or laser (700-850nm) or UV light (below 270nm), brought on by discrete changes in eminent light, above and underneath the wavelength

of the incident photons because of the vibrational frequencies of the bio-molecules that constitute the tissue (11-19).

The utilization of Raman spectroscopy has progressed into the various fields of science and physics. It is a valuable tool in biomedical field making it possible to extract the data from the spectra using a number of spectral diagnosis methods. The spectral bands of biological and synthetic tissues are specific to particular chemical groups that can be recognized and analyzed. Innovation of fiber optic-probes has made possible to examine tissue without sectioning (20-24). Benefits of Raman spectroscopy are non-invasive, non-destructive, requires minimal or no sample preparation, thus helping in faster diagnosis. Based on its other advantages, which include linear response to chemical concentrations, easier spectral/band analysis, sharp spectral signatures, weak water scattering and reproducible results, Raman spectroscopy is widely applied in biomedicine as a device for diagnosis of tissue lesions, examination of blood segments, investigation of single cells and even non-contact monitoring technology in various biomedical fields (25-45).

Furthermore, Raman spectroscopy is a helpful tool in estimating the cancer margins, which assist the surgeon in conservative excision of the cancer lesion without any remnants. Its high spatial and spectral resolution demarcates the exact location and borders of the lesion even to an extent of single molecule identification (46). Spatially Offset Raman Spectroscopy (SORS) and Transmission Raman Spectroscopy (TRS) assures diagnosis even in deeply located lesions (47). Raman spectroscopy, by adopting the Gaussian classification method, can efficiently differentiate deeply seated oral carcinomas (48). Latest modifications in the probe design has

allowed Stimulated Raman Spectroscopy (SRS) examination of superficial tissues in hollow tracts of the human body, such as oral mucosa (49).

Raman spectroscopy can also be used to reveal the molecular framework of proteins, lipids, DNA and other biological molecules, providing a "molecular fingerprint" (50). In particular, the molecular fingerprint in the range of 1800 to 100cm^{-1} which coincide with the spectra of biochemical structures provides the data of specific biochemical structures (biochemical signatures) of the tissue, providing an opportunity to distinguish between tissue types. Additionally, spectral collection can be performed in vitro or in vivo without disturbing the cellular environment, and it has the capacity of detecting minimal alterations in the biochemical composition of living cells to produce a diagnostic molecular fingerprint of the target tissue without provoking photo- induced cellular damage (45, 51).

The use of Raman spectroscopy for discriminating the malignant and the pre malignant lesions from the normal site has been studied in various locations, which include brain, breast, gastro-intestinal tract, gynecologic tract, larynx, skin and oral cavity (52-58). This article audits the uses of Raman spectroscopy in dentistry based on its distinguishable spectral characteristics to analyze healthy and pathological oral soft tissues. By recognizing the relevant peaks (Table 1), this article serves as an aide for further studies focused on application of Raman spectroscopy on oral soft tissues.

Path Toward Clinical Implications of Raman Spectroscopy

Technology Development

The technology and methodology had to be developed to collect and interpret the Raman spectra, for a considerable period of time, as tissue fluorescence and lack of applicable, sensitive

instrumentation were major obstacles for the development of Raman spectroscope for the clinical applications (12). Nevertheless, the field of Raman spectroscopy has seen tremendous advances over the last thirty years. The difficulty of tissue fluorescence, has been overcome to the large extent by the accessibility of instruments operating within the near-IR region. Fluorescence from biomolecules, cells and tissues can be avoided by laser excitation within the deep UV excitation and the robust auto fluorescence background caused by optical elements was overcome by increasing the excitation of laser (11, 12)

With the arrival of nanotechnology and its application in medicine, Surface Enhanced Raman Spectroscopy (SERS) has emerged to unravel the challenges encountered with Raman spectroscopy. SERS improves the Raman signal by 10^9-10^{14} times (45). In addition, the robust fluorescence background is greatly reduced, due to the absorption of molecules on the metal nanoparticles, there by resonance enhancement of particular molecular vibrations can be achieved (5). The SERS is a promising technique for analyzing biomolecules, as well as biomarkers, such as, deoxyribonucleic acid (DNA), cells, viruses and microorganisms for early detection of oral cancers (11). It can combat inherently weak signal from saliva by using surface enhanced approach with gold particles (10).

Applications of Raman Spectroscopy in Normal Oral Soft Tissues

The oral cavity is accepted to have an impact on systemic wellbeing. Different studies have demonstrated that oral cavity shows an important part in giving forecasts, furthermore the anticipation of fundamental systemic wellbeing (33, 59).

Raman spectroscopy helps in the screening of asymptomatic patients to recognize the concealed primary or otherwise malignant lesions and furthermore to evaluate the hidden risk of

developing cancer. There are considerable differences between well developed and synchronized histological assessment, such as, WHO dysplasia grading system and the Ljubljana grading system, which are based on the same architectural and cytological changes (60). Unfortunately, there is no simple relationship or overlapping between the classification systems, figures 1a --c show the areas of similarity in the classification systems but also the problems arising when matching the WHO categories moderate and severe dysplasia with the Ljubljana category atypical hyperplasia Figure 1d [60]. Figure 1a-d shows the development of oral cancer over a period of time; and recognition of severe dysplasia starts with greater than two-thirds of the epithelium showing architectural disturbance with associated cytological atypia. However, architectural disturbance extending into the middle third of the epithelium with sufficient cytological atypia is upgraded from moderate to severe dysplasia. Raman spectroscopy may be an effective technique to accurately detect the pre-cancerous lesions of the oral cavity, the mucosa collected is inhomogeneous consisting of different layers and histological structures. Cals FLJ et al reported on the Raman spectroscopic analysis of histopathological structures in oral mucosa (61). Raman spectroscopy also offers an attractive potential to achieve dependable and reproducible signals from various oral sites, among subjects of diverse races and gender (37).

Near Infrared (NIR) Fourier Transform (FT) Raman spectroscopy presented in the mid-80s minimized and in some cases diminished the issue of fluorescence, by using the substrates made of CaF_2 and BaF_2 and test debasement, which is chosen over visible light excitation. This particular property is responsible for the potential in vivo tissue diagnosis, because NIR wavelengths could penetrate deeper into tissue with less absorption by surrounding tissue

components (2, 12). In recent years, NIR-Raman spectroscopy has shown favorable results for the exact diagnosis and evaluation of the disease progression in various organs, especially in the oral cavity which helps to evaluate the resection margins during excision of any intraoral malignancy (62, 63). It has the ability to penetrate the tissue for several millimeters, creating a "diagnostic window" a distinguishing feature to diagnose sub-mucosal lesions (21). A number of researchers have used this approach to analyze biological tissue mounted on CaF_2 windows by Raman spectroscopy (2, 12).

Wu et al reported on the use of FT-Raman spectroscopic for the analysis of oral tissues. Well defined strong C-H stretching bands were observed at 2925 and 2959 cm^{-1} and the C = O vibration of triglycerides at 1740-1745 cm^{-1} in normal tissues, in contrast to the weak C-H stretching band and C = O band in the malignant tissue. These results demonstrated an effective, convenient, accurate and noninvasive method of cancer diagnosis (64). de Veld et al (2005) investigated the biopsy tissues of 37 human oral mucosa lesions. Complete auto-fluorescence pictures and spectra were recorded from 20 micron section areas. Study evaluated the possible relationship between spectral shape and lesion type or cell layer. Auto-fluorescence pictures demonstrated high intensities of keratin layers and connective tissue, yet scarcely any for the epithelium. This study showed clear variations between different cell layers, concluding the high sensitivity and specificity of Raman spectroscopy for demonstrating biochemical changes (58). Guze et al performed multivariate analysis on 25 Caucasian and 26 Asian patients at 7 specified sites within the oral cavity to study the dependability and reproducibility of Raman spectroscopy among distinctive races and sex. Study showing unique spectra based on location of tissue was attributed to keratin levels in the respective tissues. However, no significant spectral association

was observed for different subject ethnicity or sex. They also illustrated, the potential use of Raman spectra for in vivo cancer diagnosis (6).

Lo et al. applied Raman spectroscopy to Ex Vivo Produced Oral Mucosa Equivalent (EVPOME) to see the cellular activity. They observed a positive correlation with amide I (1655 cm^{-1})/phenylalanine (1004 cm^{-1}) with a negative linear regression ($R2 > 0.95$), particularly on the 14th and 21st culture day (65). To study the epithelium at different levels, it was sliced into various thicknesses and was found that the Raman peak intensities of amide I, C--H, amide III and C--OH decrease as the keratinocytes mature. This aspect also determined the relationship between the maturities of the keratinized oral mucosa and various components between the epithelial and connective tissue cells. They observed a relative increase in intensity of the 1254 cm^{-1} peak in the amide III region and the right shift of the wavelength in the amide I region at deeper levels. This shows the favorable application of Raman spectroscopy for quantitatively monitoring and evaluating the maturity of EVPOME.

Bergholt and Huang also used Raman spectroscopy to study oral soft tissues at different anatomical locations. Distinctive spectra related to the biochemical variations at different anatomical sites of the oral cavity were observed within the spectral range of 1800--800 cm^{-1} (66). They used specially designed fiber-optic Raman probe with a ball lens for real-time, in vivo Raman measurements at different areas in the oral cavity (i.e. inner lip, attached gingiva, the floor of the mouth, dorsal and the ventral surface of the tongue, hard and soft palate and buccal mucosa). The semi quantitative non-negativity-constrained elements (i.e. hydroxyapatite, keratin, collagen, DNA, and oleic acid) and partial least squares-discriminant analysis (PLS-DA) were employed to estimate the importance of inter-anatomical variability. This study reveals

significant inter-anatomical variability and suggested that histological and morphological attributes of different sites have a significant impact on the in vivo Raman spectra, and thus various sites can be categorized with an overall sensitivity and specificity of 85%. Their work on Characterizing variability in in-vivo Raman spectra of different anatomical locations in the upper gastrointestinal tract toward cancer detection was of interest as this study was part of an ongoing nationwide cancer screening program aiming at early diagnosis and treatment of upper GI malignancies run by the Singapore gastric cancer epidemiology, clinical and genetic program. This demonstrated that Raman spectroscopy can be very successfully employed to differentiate between actin, collagen, DNA, histones, and triolein) which are used for biochemical modeling of other cancerous tissues as well. Bergholt et al further reported that high quality in vivo Raman spectra can routinely be acquired from different anatomical locations or lesion sites within the esophagus and gastric in real-time (<0.5 s) under multimodal imaging (WLR, NBI, and AFI) guidance during clinical endoscopic examinations. [see Figure 2]. They also reported that to characterize the spectral contribution from important Raman active tissue constituents (e.g., proteins, DNA, and lipids) semi-quantitative modeling was based on five spectra of actin, collagen type I, DNA, histones, and triolein) [see Figure 3], which may also be valuable in modelling other types of cancer from different anatomical sites.

Applications of Raman Spectroscopy in Oral Cancer

The alteration of healthy tissue into a malignant lesion is a gradual process which involves changes at the molecular level, thereby altering the morphology and tissue architecture. Cancer is an unusual growth of cells, which might conceivably attack the neighboring normal cells if left untreated. Despite the fact that there are more than 200 different varieties of cancer,

the malignancy of epithelial layer accounts for 85% (67, 68). Carcinomas of the head and neck are one of the most common cancers, of which the majority originates from the oral cavity most commonly involving the buccal and labial mucosa, tongue, the floor of the mouth, gingiva, lips, periodontium and oropharynx (69, 70). The standard techniques of evaluation of any oral lesion are thorough clinical examination followed by assessing its potential in progressing to oral cancer (71, 72). Oral cancers are often diagnosed at the terminal stages associated with extensive histopathological changes, hence regardless of treatment there is a more chance of recurrence (73, 74).

In spite of the advancement in surgery and radio-chemotherapy, no huge enhancements with respect to patient survival could be seen in the most recent decades (75). It has been demonstrated that the survival rate relates profoundly with the disease stage (76). Inappropriate methods of cancer screening and diagnosis results in delayed identification of the disease (77). Thus, early diagnosis is required to avoid life threatening complications (66). Conventional diagnostic methods such as visual inspection, biopsy and histopathology inherit inter examiner variations (78). Human and economic constraints, lack of significant clinical presentation, patient reluctance to undergo biopsy, sampling mistakes, obligatory invasiveness and tissue loss associated with biopsy are the variables influencing the delay and errors of the diagnostic process (79-81). This leads to the increasing interest in finding alternative diagnostic techniques such as cytological techniques, histomorphometric analysis, cytomorphometry, DNA cytometry and immunocytochemical analysis, optical coherence tomography, high resolution micro-endoscopy (HRME) and polymerase based diagnostic kits (82, 83).

These bio-optical approaches could overcome the diagnostic restraints on the basis of a familiar principle that, when tissues are illuminated with light, the derivative of this optical spectrum will give the data about the molecular or chemical composition and the surface characteristics of the exposed tissue (38). These light- based imaging of the tissues detects even minimal changes in cell microanatomy (nuclear/cytoplasmic ratio), DNA Ploidy and quantification of nuclear DNA content, redox status, expression of specific biomarkers, tissue architecture and composition, chemical changes (mineralization), vascularity/angiogenesis and perfusion (78, 84, 85). Tissue transformation from normal to malignant state brings about changes in the morphology at molecular levels, which has an impact on the absorption and reflection of light. Delivery of a specific wavelength of light to both the normal and abnormal tissue is kept as a standard spectroscopic data and any abnormal tissue of unknown origin can be analyzed and compared with the available pattern that is histologically proved to be normal and abnormal tissue thus aiding in diagnosis (86). The detectable changes at molecular level are perfect for recognition of early changes in the lesion tissues, evaluation of lesion margins, the likely presence of subclinical margins, monitoring of the existing noninvasive lesions and brisk examination of at risk population (87). Various spectroscopic techniques have their own spectral patterns of different tissues with varying normal, precancerous and malignant changes. These specific patterns help in assessing the stage of carcinogenesis, (86) and this collected information gives a scope for characterization of the tissue by a number of bio-optical methods, of which Raman spectroscopy is one of the most optimal method to be discussed in this particular paper, which would provide detailed biochemical information that may be used to detect initial stages of malignancy. The Raman bands noticed during this maneuver that help in diagnosis are mainly

relevant to proteins, possibly collagen (tyrosine, tryptophan, and phenylalanine), nucleic acids, and lipids. The developments in the application of Raman spectroscopy by the use of near infrared excitation decreased the autofluorescence of biological specimen, whereas, the fiber-optic Raman probes contributed in molecular fingerprinting and in vivo analyses. Currently, automated Raman systems are being employed in rapid evaluation of the biological tissues in vivo, very often within 5 sec or less (4, 6, 21, 30).

Malini et al in 2006 (24) analyzed the competence of Raman spectroscopy to segregate 216 pre-malignant, malignant, inflammatory and normal tissue samples from the oral cavity. In spite of having accessibility to oral cavity, the majority of lesions are diagnosed in last stages of the illness, to be more precise unfortunately around 60 percent of them were in T3 or T4 by the time they were diagnosed. This indicates a critical impact on the survival rate, which is 40% according to the World Health Organization (69) or less than 60% according to American Cancer Society (39). In this scenario, Raman spectroscopy may prove to be a useful adjunctive tool for early diagnosis, which is of paramount importance to the long-term survival of patients.

The oral cavity is easily approachable in a clinic setting and would be a perfect model for the development of a Raman probe for cancer detection. Raman spectroscopy explicit the progressions in molecular composition and molecular adaptation that happen in tissue amid carcinogenesis. Recent advancements in fiber-optic test innovation empower its application as an in vivo procedure (68) with the exception of its use in parotid tumors because of the impedance of the superficial skin and subcutaneous tissues, for which only ex vivo is advised. Modern advancements in Raman spectroscopic instrumentation and data management analysis have prompted challenging results concerning the spectroscopic diagnosis of cyto-pathological

samples. Furthermore, the freedom of labeling and staining has allowed the recognition of circulating malignant cells in body fluids (88). Recent studies reveal that Raman spectroscopy disclosed promising results in identification of pathologies of head and neck regions (10, 21). Studies have shown the practicality of the use of Raman spectroscopy in identification of oral squamous cell carcinomas (24, 61), the malignant changes of which correspond to the increase in DNA and proteins (73). Raman spectroscopy is sensitive to a wide variety of proteins, lipids and nucleic acids and has been applied in the analysis of various normal and diseased tissues of the human body including the bladder, hypopharynx, breast and testicles; and also in the evaluation of anti-cancer drugs at cellular level. (89-94).

Stone et al., evaluated utilization of Raman spectroscopy in diagnosing malignancy of larynx (56). They utilized homogenous biopsy specimens with distinctly characterized pathological findings for histopathological analysis. Study included 7 histologically normal healthy samples, 4 displaying dysplasia, 4 with carcinoma and 5 to 12 spectra were obtained from each specimen. Results showed apparent variations in the spectra in the range of 950 to 850 cm^{-1}, and 1350 to 1200 cm^{-1}. The later peaks are precisely associated with protein compliance and C-H bond in nucleic acid bases and the relative intensity of the nucleic acid peak rises with the progress of malignancy. Linear discriminant analysis showed greater extent of certainty of disease in comparison to the empirical peak ratio methods. Results achieved from cross-validation of the discriminant model revealed prediction specificities of 94%, 91%, and 90% and sensitivities of 83%, 76%, and 92% for healthy, dysplastic and carcinoma samples respectively.

Bakker Schut et al., investigated the effectiveness of Raman spectroscopy for in vivo classification of normal and cancerous tissue of rats (28). Dysplasia in the palate of rats was

incited by the topical use of a cancer-causing agent (4-nitroquinalone 1-oxide). Normal and dysplastic tissues were collected in vivo by a fiber-optic test which was coupled to a Raman spectrometer with a NIR excitation laser. Raman spectra obtained from these tissues using integration tie of 100s were tested with the in vivo spectra obtained using signal collection times of 10s, to categorize the tissues as normal, low and high grade dysplastic. The high grade dysplastic tissues were distinguished with a sensitivity and specificity of 100%.

Venkatakrishna et al., evaluated 25 biopsy samples and 24 surgically sectioned samples of which 37 were squamous cell carcinoma and the remaining normal and performed principal component analysis of 140 spectra to derive statistically significant quantitative information about the spectral components of the malignant and normal tissues. These spectral characteristics thus obtained helps in the screening of the population, decision of surgical boundaries, follow up for regression and recurrence and also progress during chemotherapy (95).

A study was conducted by Krishna et al., on the formalin fixed normal and carcinoma tissues of oral squamous epithelium stored at room temperature for a period of two months. Raman spectroscopic analysis was done on both the epithelial and the sub epithelial regions of these tissue samples. In the epithelial region of both the normal and the malignant layer, the spectra showed drastic changes in the biochemical structure and no differences were seen in the sub epithelial layer. Spectral change was attributed to the protein composition, structural change and increase in the protein content. The discrimination of normal, malignant and sub epithelial tissues was well appreciated with this technique and was significantly conclusive in validation of formalin fixed tissues for optical analysis (96).

Lau et al., obtained Raman spectra of normal, benign, and cancerous laryngeal tissues and reported chemical differences that are present between these different tissues (21). A total of 47 laryngeal samples including normal tissue, carcinoma and squamous papilloma (n = 18, 13, and 16 respectively) were studied with signal acquisition times (SAT) around 1 and 30 seconds. Multivariate investigation was performed to decide the diagnostic capacity of Raman spectra in comparison to the normal histology. Great quality of spectra were acquired with 5-second SAT. Spectral peak examination demonstrated prediction sensitivities of 89%, 69%, and 88%, and specificities of 86%, 94%, and 94% for normal tissue, carcinoma, and papilloma respectively.

Oliveira et al., used FT-Raman spectroscopy to evaluate the changes in the vibrational bands of normal, dysplastic and squamous cell carcinoma tissues. They reported that detection of weak Raman signals from tissue samples was easy by FT-Raman due to fluorescence suppression. In this study 7, 12-dimethybenzanthracene, a toxin was applied daily in the oral pouch of 21 hamsters for 14 weeks to bring about carcinogenic changes, followed by collection of normal squamous cell carcinoma tissues from the oral region and analysis using a 1064nm Nd:YAG laser as an excitation source. Around 123 spectral sequences were obtained and were grouped into normal and malignant tissue by performing statistical analysis using principal component analysis (PCA) and was also classified using Mahalanobis distance approach. Considerable differences were seen between the normal and malignant spectra, which was attributed to the composition and structural alteration in protein and increased protein content in malignant epithelium. An algorithm was based on PCA with sensitivity of 91% and 100%, and specificity of 69% and 55% in the training and prospective groups respectively. It was also

interpreted that principal component analysis had the potential for classifying Raman spectra and could be used for detection of dysplastic and malignant oral lesion (29).

Li et al., analyzed squamous cell carcinoma (SCC) of oral mucosa using near infrared Raman spectroscopy (25). Study was done on 10 normal mucosa, 20 SCC and 30 oral leukoplakia tissues to compare with the characteristic band obtained in previous studies. Classification and diagnostic models were done on Gaussian radial basis function support vector machine, and efficacy and validity of the algorithm was evaluated. Increased peak intensity in the wave number range of 2200 to 500cm^{-1} indicated high contents of DNA, protein and lipid in SCC, which also elucidated the high proliferative activity in the cell. It was observed that the discrepancy between OLK and SCC was higher when correlated to that of the difference between normal and SCC. The Gaussian radial basis function support vector machine presented effective ability in grouping and modeling of normal and SCC, and the specificity, sensitivity and accuracy were 100%, 97.44% and 98.81% respectively. This algorithm also showed good ability in grouping and modeling of OLK and SCC, with specificity of 95%, sensitivity of 86.36 and accuracy of 96.30%. It was concluded that NIR-Raman spectroscopy could delineate the biochemical differences in oral normal, OLK and SCC and establish a diagnostic model with good accuracy.

Guze et al., in 2012 employed micro-Raman spectroscopy to describe the molecular/spectral differences between healthy and squamous cell carcinoma (SCC) in oral mucosa (97). Samples confirmed by histopathological examination as squamous cell carcinomas in various grades of malignancies (SCCs) were collected from the lateral aspect of the tongue. Normal tissue from contralateral intraoral site was also collected. All the collected samples were

flash-frozen and cut into two sections, one specimen being examined histologically and the other micro-Raman (confocal) spectroscopy. Freshly flashed-frozen tumor and matched normal tongue specimens were studied from 4000 to 300 cm^{-1} spectral range. The spectral resolution used was 8 cm^{-1}, spatial resolution was 1.0μm and the excitation wavelength was 515nm. They observed statistically significant spectral differences between healthy and malignant samples in relation to the origins of specific signals within the cells of each tissue type. They discovered specific cell surface protein spectral biomarkers at 1583 cm^{-1}.

Devpura et al. (98) performed principle component and discriminant functional analysis to study normal, invasive squamous cell carcinoma and carcinoma in situ of the tongue after the samples were placed in formalin and dewaxed. The dewaxing of the fixed tissues is of utmost importance, as contamination with dust particles or wax as a result of inappropriate de-waxing affects the spectrum leading to false information and discouraging results. As wax contributes to the fingerprint region of the spectra, it interferes with amide I, keratin, nucleic acid and amide III compartments and prevent their expression, which has the most sensitive information in biological tissues. Mian et al (99) reported that in spectroscopic studies it is extremely important to completely eliminate wax in order to acquire comprehensive expression of vital components, which are of diagnostic significance. Complete removal of wax is essential to achieve spectral contributions from amide I, keratin, amide III, nucleic acid (DNA) and phenylalanine. These regions were hindered in spectra of tissues kept in xylene for up to 28 minutes whereas their expressions were recorded clearly in the spectrum analysed after 30 minutes in xylene. Wax contamination in Raman spectra obstructs the information regarding chemical composition in biological tissues. A phenylalanine contribution at 1002 cm^{-1} is expressed in the spectrum of

tissue section kept in xylene for 30 minutes, which was absent in pure wax spectrum (figure 4). Therefore, it is extremely important to eliminate wax in order to achieve accurate results. Mian et al (99) demonstrated that to eliminate wax contamination from 20µm thick tissue sections a 30 minutes treatment in xylene is required.

The goal of Devpura et al (98) study was to explore the effectiveness of Raman spectroscopy for early diagnosis and identify possible biomarkers of head and neck squamous cell carcinoma. They compared Raman spectroscopy findings with the results of histological examination and detected an accuracy of 89% for invasive squamous cell carcinoma, 91% for carcinoma in situ and normal tissues. This study also showed escalation of tryptophan values and declining of keratin in squamous cell carcinoma.

Lloyd et al. in 2013 conducted multivariate analysis to discriminate benign, primary and secondary malignancies in lymph nodes of head and neck, in which they collected 103 lymph node samples from 23 patients undergoing surgery for incredulous lymph nodes (100). Five pathological conditions defined by histopathology were studies which included benign reactive nodes, Hodgkins and Non-Hodgkins lymphoma, metastasis from squamous cell carcinoma and adenocarcinomas. Raman spectra were obtained with 830nm excitation laser and analyzed using principal component linear and partial least square discriminant analysis. The results showed sensitivity and specificity of 90 and 86% in two group models, above 78 and 89% for three-group models and above 81 and 89% for all groups.

Knipfer et al. examined 12 *ex-vivo* oral cancer samples using principal component and linear discriminant analysis after being subjected to Shifted Excitation Raman Difference Spectroscopy (SERDS). They compared the results of Raman spectroscopy and histopathology.

Mean of 72 spectra obtained from 3 physiological tissue sites and pathological lesions were examined (101). Spectra of malignant and benignant tissues were differentiated based on spectral features of proteins, lipids and nucleic acids and the results showed a classification error of 9.7%.

Guze et al. analyzed the tissue based molecular signatures of Raman spectra to differentiate the premalignant and malignant condition of normal mucosa and benign lesions, with the aim to overcome the subjective errors associated with histopathology. 18 patients with histologically diagnosed oral disease were studied and compared with Raman spectroscopy along with the contra-lateral healthy areas. Principal component and multivariate analysis were used to compare the whole spectra instead of single Raman peaks. The results showed a clear variation between premalignant and malignant lesions and healthy and benign lesions (39).

Singh and Krishna performed a study using fiber optic probe along with a Raman spectroscopy on histologically confirmed tumor of buccal mucosa and noticed higher lipid and lower protein bands in normal tissues, whereas, higher protein bands were noticed in tumor tissues. Biochemical estimation also showed similar results with high protein-to-lipid or protein to phospholipid ratio in tumors in comparison to normal tissues. The results were statistically significant at the intensity of 1440 cm^{-1} (lipid) and 1450 and 1660 cm^{-1} (protein). They concluded that, the findings of curve deconvolution and biochemical evaluation associate well and proposed that spectral features are the trademark of underlying biochemical tissue composition (102). Krishna et al also conducted a pilot study for differential diagnosis of cancerous and precancerous lesions of oral cavity in a clinical setting using *in-vivo* Raman spectroscopy (30). They studied 28 healthy volunteers and 171 patients with various oral lesions. Raman spectra was obtained from multiple different sites of normal and abnormal oral mucosa

and were categorized histopathologically as healthy, oral squamous cell carcinoma, oral submucous fibrosis and leukoplakia. The spectra was subjected to probability based multivariate statistical algorithm and found an accuracy of 85%, 89%, 85% and 82% in classifying the oral tissues.

Daniel et al. created pseudo color Raman maps from the Raman micro images of cancerous and noncancerous samples (67). They found that, the maps correlated with the histopathological image of the same tissue section with 98.9% accuracy. Study proposed a classification model to distinguish healthy and cancerous oral tissues based on the Raman spectral features. The results of this study also showed decreased presence of glycogen, lipids, and proteins, such as, tryptophan and phenylalanine in all the cancerous samples. Whereas, the nucleic acid (DNA/RNA) content was found to be less in normal samples compared to the cancerous samples (67). Shyam Sunder et al. also differentiated oral tumors of different grades using relative intensities of bands associated with lipids and proteins. They noticed an increase in proteins and decrease in lipids in malignant and normal tissues respectively (104). These results were in par with Mahadevan - Jensen et al study suggesting that Raman spectrum can effectively distinguished to the molecular and structural changes associated with neoplastic transformation (55).

Singh et al. evaluated the potential capability and efficiency of Raman spectroscopy in identifying early premalignant conditions (105). Spectra were obtained from suspected premalignant lesions, contralateral normal tissue and cancer lesion sites from subjects with oral cancers; and healthy subjects of the same age group with and without tobacco consumption habits. Study employed fiber optic probe in conjunction with Raman spectrophotometer, and

results were analyzed using principal component and linear discriminant analysis. They concluded that the premalignant conditions could be discriminated from contralateral normal and abnormal sites, as well as from healthy controls, which further supported the application of Raman spectroscopy in diagnosing oral tumors.

Singh et al also reported on malignancy associated metachronous and synchronous secondary tumors in oral cavity (106). Low survival rates in cancer afflicted individuals were attributed to malignancy associated changes in the uninvolved areas. This study employed Raman spectra to categorize five groups namely - healthy controls without any habits, healthy controls with tobacco habit, no cancer, cancer and contralateral normal, and cancer people without any habits. Spectra suggested changes in protein, lipids and DNA in pathology prone areas. There was a difference in the spectra obtained from healthy controls and those with habits and disease free, indicating that malignancy associated changes can be identified. It was concluded that Raman spectroscopy had the potential of identifying metamorphic changes in oral mucosa thus helping in early diagnosis of oral cancer.

Lau et al distinguished normal and cancerous nasopharyngeal tissue by employing a rapid acquisition Raman spectrometer (107). They collected normal and malignant biopsy tissues from 6 patients and the obtained spectra showed, reliable differences between normal and malignant tissue in three bands 1320 -- 1290cm-^{1} (P¼0.005), 1470 -- 1420cm^{-1} (P¼0.006), and 1580 -- 1530cm^{-1} (P¼0.002). A similar study was done by Feng et al with gold nanoparticle based surface-enhanced Raman scattering spectroscopy by employing near-infrared laser excitation and concluded that amino acids decrease in cancerous conditions, whereas the nucleic acids (DNA) and thymine increases (108). Huang found that the combination of nanorods could be

advantageous instead of spherical gold colloids. They noticed that human oral cancer cells are kept together and arranged next to gold nanorods that were combined with antibodies against the epidermal growth factor receptor. The molecules located near nanorods produced an incredibly improved, sharp, and polarized Raman spectrum. The authors have demonstrated that these nanorods have the capacity to be utilized as cancer biomarkers (68).

Application of Raman Spectroscopy on Bio-fluid Diagnostic Assays

The investigation of bio-fluids can be used as diagnostic assays in a variety of medical conditions, ranging from routine blood check up to intra operative monitoring due to its easy accessibility, less invasiveness, and its ability to allow for repeated sampling. Body fluids contains biomolecules which are of diagnostic significance, but at times, in inadequate concentration to be estimated with Raman spectroscopy. One highly efficient way is to pre-concentrate the protein for drop coated deposition Raman spectroscopy (DCDRS). This has been shown to provide a precise estimation of the concentration of protein mixtures in solutions even when pre-concentrated. The use of DCDRS has been exhibited in human tears indicating a promising tool for the measurement of both local and systemic disease biomarkers. Discovery of genetic and epigenetic materials arising from tumor or circulating tumor cells in body fluids symbolize an attractive option for quicker and non-invasive identification of early cancers (109).

Harris et al. explored to discriminate the noncancerous and cancerous lesions by Raman spectroscopy using a peripheral blood sample. They collected 40 blood samples from patients suffering from head and neck cancers and patients with respiratory illness to be used as control group (110). Principal component and linear discriminant analysis achieved approximately 65% sensitivity and specificity for differentiating cancer and respiratory illness in samples.

Furthermore, when the genetic program was used to the data, an increased sensitivity and specificity of 75% was seen. Based on this pilot study, they concluded the possible feasibility of using Raman spectroscopy in screening and diagnosis of cancers and solid tumors through a peripheral blood sample. However, it is premature to make any positive conclusions on the basis of this limited study.

The metabolic products released into the circulatory system from cells or tissues as end products that alter in systemic conditions may serve as markers for that particular condition. Based on these markers, Sahu et al examined serum from patients suffering from oral cancer of buccal mucosa and tongue, and healthy patients (111). Spectral features indicate diverse contributions of proteins, DNA, and amino acids like Phenylalanine, Tryptophan and Trypsin, and β-carotene in the selected groups. Profound Raman bands on β-carotene were observed in normal patients and increased DNA and proteins were observed in cancer spectra unlike the phenylalanine decrease in cancer patients. Additionally, a propensity of categorizing buccal mucosa and tongue cancers was noticed and the study reported a diagnostic sensitivity and specificity of 81% and 75% in the detection of oral cancer by using 514nm excitation laser.

Rekha et al identified the biomolecules present in the blood plasma in a clinically confirmed healthy normal, precancerous and cancerous groups by using 784nm laser excitation (112). Alternative to serum, saliva was taken into consideration by Qiu et al. in detection of oral cancer (113). Because 95% of the saliva is water, a surface enhanced Raman or SERS was utilized to identify the delicate chemical alterations in salivary samples. The uses of saliva in diagnosing the cancer have few advantages which include easy, painless, readily accessible, non-invasive, quick, and economical sample collection (114, 115). According to Olivo M the

peaks at 1627, 1079 and 670 cm^{-1} have important diagnostic value and especially the peak at 1627 cm^{-1} provides valuable data indicating malignant changes using saliva specimens (116). Qiu et al. evaluated the use of saliva SERS for detection of non-invasive nasopharyngeal carcinoma. Analysis showed significant differences at 1448, 1270, 1134, 729, 635, 496 and 447 cm^{-1}, which basically consist of signals related to collagen, glycogen, fatty acids, nucleic acids and proteins. They provided a relatively high diagnostic sensitivity, specificity and accuracy of 86.7%, 81.3% and 83.9% respectively. The SERS peak centered at 729 cm^{-1} demonstrates highest intensity in nasopharyngeal carcinoma patients indicating the amount of DNA and RNA in saliva due to the apoptosis of cancerous cells and tumor necrosis (114).

Applications of Raman Spectroscopy in Other Conditions

Sialoliths associated with the salivary glands and their ductal system are calcified masses usually composed of calcium phosphate (hydroxyapatite: $[Ca_{10}(PO_4)_6(OH)_2]$ with traces of magnesium and carbonate. These mineral components have been studied by various methods including high-resolution electron microscopy and X-ray diffraction. Kinoshita et al. explained in detail the precise formation of sialolith by analyzing the actual distribution of organic (amide III) and inorganic (phosphate) content within the sialolith with the help of Raman spectroscopy (117). The sialolith from the submandibular gland was used for the study. They observed the spectral peaks of amide III and phosphate at 1265 and 960 cm^{-1} respectively (118).

Yan et al. proposed a technique to apply Raman spectroscopy to examine the differences in the spectra between Warthin's tumor, pleomorphic adenoma, and normal parotid gland tissues (41). Along with this, they setup the diagnostic model of the Raman spectra of parotid gland neoplasms by using support vector machine (SVM) with Gaussian radial basis function. SVM

was used to examine the different Raman spectra obtained from these various histopathological tissues. Results showed difference in the spectral region of 1800 to 800cm^{-1} demonstrating the biochemical alteration at the molecular level among the histopathological tissues. In comparison with normal parotid gland tissues, there was rise in the content of proteins, lipids and DNA in pleomorphic adenoma. Where as in Warthin tumor the content of DNA was increased but proteins and lipids were decreased. Yan et al. also demonstrated research to detect label-free blood serum by using surface-enhanced Raman spectroscopy (SERS) and support vector machine (SVM) in the pre-operative evaluation of parotid gland tumors (117).

In addition to the in vivo applications, Raman spectroscopy offers numerous open doors for the advancement of other sensitive diagnostic tools, quick labeling of pathogenic micro-organisms and tumor grade typing (5,12). Raman spectroscopy empowers to identify the most frequent pathogenic microorganisms in the circulatory system infections in patients admitted in intensive care units (ICU). Very quick labelling and testing the susceptibility, and quick reporting paves the way for the selection of most appropriate antibiotics in cases of infection, and the most suitable therapy in early stages of cancer (12).

Summary

Raman spectroscopy is rapidly becoming an established technique for the chemical structural analysis of soft tissues, as a number of clinical biopsies and non-clinical samples (tissue engineered samples) can be analyzed with minimal sample preparation and furthermore the drug interactions with the tissue can also be studies in detail. In addition to its use as a diagnostic technique, it can be an excellent tool to monitor the progression of disease or to identify the resistant and sensitive nature of the drug employed to treat cancer. Raman

spectroscopy had shown promising results with the added advantage of being a non-invasive, non-destructive tool along with providing a real time diagnosis and due to the enormous leap in using Raman spectroscopy for diagnosis, it is at a stage to perform systematic clinical trials for diagnosing a dysplastic condition in a very early stage of cancer development.

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References

1. Max, D., Kathleen, L, Jen, S., Ben, B., Milo, M., Krafft, C. and Popp, J. (2013). Molecular pathology via IR and Raman spectral imaging. *J. Biophotonics.,* 6: 855-886.
2. Krafft, C. and Popp, J. (2014). Vibrational Spectroscopic Imaging of Soft Tissue. In *Infrared and Raman Spectroscopic Imaging* 2nd ed. Wiley-VCH Verlag GmbH & Co: Weinheim, Germany.
3. Krishnan, K. S. and Raman, C. V. (1928). A new type of secondary radiation *Nature.,* 121, 501-502.
4. Hanlon, E.B., Manoharan, R., Koo, T.W., Shafer, K.E., Motz, J.T., Fitzmaurice, M., Kramer, J.R., Itzkan, I., Dasari, R.R., and Feld, M.S. (2000). Prospects for in vivo Raman spectroscopy. *Phys. Med. Biol.,* 45: R1-59.
5. Brauchle, E., and Schenke-Layland, K. (2013). Raman spectroscopy in biomedicine - non-invasive in vitro analysis of cells and extracellular matrix components in tissues. *Biotechnol. J.,* 8: 288-297.
6. Guze, K., Short, M., Sonis, S., Karimbux, N., Chan, J., and Zeng, H. (2009). Parameters defining the potential applicability of Raman spectroscopy as a diagnostic tool for oral disease. *J. Biomed. Opt.,* 14: 014016.
7. Peterson, D.E. and Lalla, R.V. (2010). Oral mucositis: the new paradigms. *Curr. Opin. Oncol.,* 22: 318-322.
8. Singh, S.P., Ibrahim, O., Byrne, H.J., Mikkonen, J.W., Koistinen, A.P., Kullaa, A.M., and Lyng, F.M. (2015). Recent advances in optical diagnosis of oral cancers: Review and future perspectives. *Head. Neck.,* doi: 10.1002/hed.24293. 1-9.

9. Hughes, O.R., Stone, N., Kraft, M., Arens, C. and Birchall, M.A. (2010). Optical and molecular techniques to identify tumor margins within the larynx. *Head. Neck.,* 32: 1544-1553.

10. Harris, A.T., Rennie, A., Waqar-Uddin, H., Wheatley, S.R., Ghosh, S.K., Martin-Hirsch, D.P., Fisher, S.E., High, A.S., Kirkham, J. and Upile, T. (2010). Raman spectroscopy in head and neck cancer. *Head. Neck. Oncol.,* 2: 26.

11. Omar, E. (2015). Current concepts and future of noninvasive procedures for diagnosing oral squamous cell carcinoma--a systematic review. *Head. Face. Med.,* 11: 6.

12. Kong, K., Kendall, C., Stone, N. and Notingher, I. (2015). Raman spectroscopy for medical diagnostics--From in-vitro biofluid assays to in-vivo cancer detection. *Adv. Drug. Deliv. Rev.,* 89: 121-134.

13. Tsuda, H. and Arends, J. (1997). Raman spectroscopy in dental research: a short review of recent studies. *Adv. Dent. Res.,* 11: 539-547.

14. Swinson, B., Jerjes, W., El-Maaytah, M., Norris, P. and Hopper, C. (2006). Optical techniques in diagnosis of head and neck malignancy. *Oral. Oncol.,* 42: 221-228.

15. Brauchle, E., Thude, S., Brucker, S.Y. and Schenke-Layland, K. (2014). Cell death stages in single apoptotic and necrotic cells monitored by Raman microspectroscopy. *Sci. Rep.,* 4: 4698.

16. Votteler, M., Carvajal Berrio, D.A., Pudlas, M., Walles, H. and Schenke-Layland, K. (2012). Non-contact, label-free monitoring of cells and extracellular matrix using Raman spectroscopy. *J. Vis. Exp.,*29: doi: 10.3791/3977.

17. Andrade, P.O., Bitar, R.A., Yassoyama, K., Martinho, H., Santo, A.M., Bruno, P.M. and Martin, A.A. (2007). Study of normal colorectal tissue by FT-Raman spectroscopy. *Anal. Bioanal. Chem.,* 387: 1643-1648.

18. Choo-Smith, L.P., Edwards, H.G., Endtz, H.P., Kros, J.M., Heule, F., Barr, H., Robinson, J.S., Jr., Bruining, H.A. and Puppels, G.J. (2002). Medical applications of Raman spectroscopy: from proof of principle to clinical implementation. *Biopolymers.,* 67: 1-9.

19. Bohorfoush, A.G. (1996). Tissue spectroscopy for gastrointestinal diseases. *Endoscopy.,* 28: 372-380.

20. Mahadevan-Jansen, A. and Richards-Kortum, R.R. (1996). Raman spectroscopy for the detection of cancers and precancers. *J. Biomed. Opt.,* 1: 31-70.

21. Lau, D.P., Huang, Z., Lui, H., Anderson, D.W., Berean, K., Morrison, M.D., Shen, L. and Zeng, H. (2005). Raman spectroscopy for optical diagnosis in the larynx: preliminary findings. *Lasers. Surg. Med.,* 37: 192-200.

22. Choo-Smith, L. P., Hewko, M. and Michael, G. S. (2010). Emerging Dental Applications of Raman Spectroscopy. In Emerging Raman Applications and Techniques in Biomedical and Pharmaceutical Fields. Springer-Verlag: Berlin, Heidelberg, pp. 263.

23. Deshmukh, A., Singh, S.P., Chaturvedi, P. and Krishna, C.M. (2011). Raman spectroscopy of normal oral buccal mucosa tissues: study on intact and incised biopsies. *J. Biomed. Opt.,* 16: 127004.

24. Malini, R., Venkatakrishna, K., Kurien, J., Pai, K.M., Rao, L., Kartha, V.B. and Krishna, C.M. (2006). Discrimination of normal, inflammatory, premalignant, and malignant oral tissue: a Raman spectroscopy study. *Biopolymers.,* 81: 14.

25. Li, Y., Wen, Z.N., Li, L.J., Li, M.L., Zhang, Z. and Gao, N. (2010). [Near infrared Raman spectral character and diagnostic value of squamous cell carcinoma of oral mucosa]. *Hua. Xi. Kou. Qiang. Yi. Xue. Za. Zhi.,* 28: 61-64.

26. Nirmal, R., Kartha, N., Ullas, V. B. and Kurian, G. J. (2011). Laser Raman spectroscopy: a novel diagnostic tool for oral cancer. *J. Orofac. Sci.,* 3: 15-19.

27. Devpura, S., Thakur, J. S., Sethi, S., Naik, V. M. and Naik, R. (2012). Diagnosis of head and neck squamous cell carcinoma using Raman spectroscopy: tongue tissues. *J. Raman. Spectrosc.,* 43: 7.

28. Bakker Schut, T.C., Witjes, M.J., Sterenborg, H.J., Speelman, O.C., Roodenburg, J.L., Marple, E.T., Bruining, H.A. and Puppels, G.J. (2000). In vivo detection of dysplastic tissue by Raman spectroscopy. *Anal. Chem.,*72: 6010-6018.

29. Oliveira, A.P., Bitar, R.A., Silveira, L., Zangaro, R.A. and Martin, A.A. (2006). Near-infrared Raman spectroscopy for oral carcinoma diagnosis. *Photomed. Laser. Surg.,* 24: 348-353.

30. Krishna, H., Majumder, S.K., Chaturvedi, P., Sidramesh, M. and Gupta, P.K. (2014). In vivo Raman spectroscopy for detection of oral neoplasia: a pilot clinical study. *J. Biophotonics.,* 7: 690-702.

31. Short, M.A., Lam, S., McWilliams, A., Zhao, J., Lui, H. and Zeng, H. (2008). Development and preliminary results of an endoscopic Raman probe for potential in vivo diagnosis of lung cancers. *Opt. Lett.,* 33: 711-713.

32. Chau, A.H., Motz, J.T., Gardecki, J.A., Waxman, S., Bouma, B.E. and Tearney, G.J. (2008). Fingerprint and high-wavenumber Raman spectroscopy in a human-swine coronary xenograft in vivo. *J. Biomed. Opt.,* 13: 040501.

33. Ramakrishnaiah, R., Rehman, G. U., Santhosh, B., Al Khuraif, A. A., Durgesh, B. H., Samad Khan, A. and Rehman, I. U. (2015). Applications of Raman Spectroscopy in Dentistry: Analysis of Tooth Structure. *Appl. Spectros. Rev.,* 50: 18.

34. de Boer, E., Moore, L.S., Warram, J.M., Huang, C.C., Brandwein-Gensler, M.S., van Dam, G.M., Rosenthal, E.L. and Schmalbach, C.E. (2015). On the horizon: Optical imaging for cutaneous squamous cell carcinoma. *Head. neck.,* doi: 10.1002/hed.24079.

35. Sigurdsson, S., Philipsen, P.A., Hansen, L.K., Larsen, J., Gniadecka, M. and Wulf, H.C. (2004). Detection of skin cancer by classification of Raman spectra. *IEEE. Trans. Biomed. Eng.,* 51: 1784-1793.

36. Xue, L., Sun, P., Ou, D., Chen, P., Chen, M. and Yan, B. (2014). Diagnosis of pathological minor salivary glands in primary Sjogren's syndrome by using Raman spectroscopy. *Lasers. Med. Sci.,* 29: 723-728.

37. Christian, K., Johanna, M., Werner, A., Kathrin, B., Tesfay, G.M., Robert, H., Abbas, A., Stefan, W., Andreas, B., Wilhelm, N.F., et al. (2014). Raman difference spectroscopy: a non-invasive method for identification of oral squamous cell carcinoma. *Biomed. Opt. Express.,* 5: 3252-3265.

38. Puppels, G. J. (2002). Preface to the special issue on medical applications of Raman spectroscopy. *J. Raman. Spectrosc.,* 33: 496-497.

39. Guze, K., Pawluk, H.C., Short, M., Zeng, H., Lorch, J., Norris, C. and Sonis, S. (2015). Pilot study: Raman spectroscopy in differentiating premalignant and malignant oral lesions from normal mucosa and benign lesions in humans. *Head. neck.,* 37: 511-517.

40. Silveira, F.L., Pacheco, M.T., Bodanese, B., Pasqualucci, C.A., Zangaro, R.A. and Silveira, L., Jr. (2015). Discrimination of non-melanoma skin lesions from non-tumor human skin tissues in vivo using Raman spectroscopy and multivariate statistics. *Lasers. Surg. Med.,* 47: 6-16.

41. Yan, B., Li, Y., Yang, G., Wen, Z.N., Li, M.L. and Li, L.J. (2011). Discrimination of parotid neoplasms from the normal parotid gland by use of Raman spectroscopy and support vector machine. *Oral. Oncol.,* 47: 430-435.

42. Salzer, R., Steiner, G., Mantsch, H.H., Mansfield, J. and Lewis, E.N. (2000). Infrared and Raman imaging of biological and biomimetic samples. *Fresenius. J. Anal. Chem.,*366: 712-716.

43. Movasaghi, Z., Rehman, S. and Rehman, I.U. (2008). Fourier transform infrared (FTIR) spectroscopy of biological tissues. *Appl. Spectros. Rev.,* 43: 45.

44. Krafft, C., Dietzek, B. and Popp, J. (2009). Raman and CARS microspectroscopy of cells and tissues. *Analyst.,* 134: 1046-1057.

45. Yasser, M., Shaikh, R., Chilakapati, M.K. and Teni, T. (2014). Raman spectroscopic study of radioresistant oral cancer sublines established by fractionated ionizing radiation. *PloS. one.,* 9: e97777.

46. Zhang, R., Zhang, Y., Dong, Z.C., Jiang, S., Zhang, C., Chen, L.G., Zhang, L., Liao, Y., Aizpurua, J., Luo, Y., Yang, J. L. and Hou, J. G. (2013). Chemical mapping of a single molecule by plasmon-enhanced Raman scattering. *Nature.,* 498: 82-86.

47. Matousek, P. (2010). Spatially Offset Raman Spectroscopy-Emerging Concepts and Applications. *AIP Conference Proceedings*, Boston, USA, August 8-10, pp 16-17.

48. Matousek, P. and Stone, N. (2013). Recent advances in the development of Raman spectroscopy for deep non-invasive medical diagnosis. *J. Biophotonics.,* 6: 7-19.

49. Mittal, R., Balu, M., Krasieva, T., Potma, E.O., Elkeeb, L., Zachary, C.B. and Wilder-Smith, P. (2013). Evaluation of stimulated Raman scattering microscopy for identifying squamous cell carcinoma in human skin. *Lasers. Surg. Med.,* 45: 496-502.

50. Lili, X., Bing, Y. and Yi, L. (2015). Application of Raman spectroscopy in the stomatology. *Hua. Xi. Kou. Qiang. Yi. Xue. Za. Zhi.,* 33: 214-217.

51. Puppels, G.J.e.a. (1991). Laser irradiation and Raman spectroscopy of single living cells and chromosomes: Sample degradation occurs with 514.5 nm but not with 660 nm laser light. *Exp. Cell. Res.,* 195: 7.

52. Talari, ACS., Evans, CA ., Holen, I., Coleman, RE., and Rehman, IU.; May (2015) Raman Spectroscopic Analysis Differentiates Between Breast Cancer Cell Lines; Journal of Raman Spectroscopy; Volume 46, Issue 5, pages 421–427,

53. Alfano, R.R., Liu, C. H., Sha, W. L., Zhu, D., Akins, L., Cleary, J., Prudente, R. and Cellmer, E. (1991). Human breast tissues studied by IR Fourier transform Raman spectroscopy. *Laser. Life. Sci.,* 4: 23-28.

54. Chen, Y., Dai, J., Zhou, X., Liu, Y., Zhang, W. and Peng, G. (2014). Raman spectroscopy analysis of the biochemical characteristics of molecules associated with the malignant transformation of gastric mucosa. *PloS. one.,* 9: e93906.

55. Mahadevan-Jansen, A., Mitchell, M.F., Ramanujam, N., Malpica, A., Thomsen, S., Utzinger, U. and Richards-Kortum, R. (1998). Near-infrared Raman spectroscopy for in vitro detection of cervical precancers. *Photochem. Photobiol.,* 68: 123-132.

56. Stone, N., Stavroulaki, P., Kendall, C., Birchall, M. and Barr, H. (2000). Raman spectroscopy for early detection of laryngeal malignancy: preliminary results. *Laryngoscope.,* 110: 1756-1763.

57. Gniadecka, M., Wulf, H.C., Nielsen, O.F., Christensen, D.H. and Hercogova, J. (1997). Distinctive molecular abnormalities in benign and malignant skin lesions: studies by Raman spectroscopy. *Photochem. Photobiol.,* 66: 418-423.

58. de Veld, D.C., Bakker Schut, T.C., Skurichina, M., Witjes, M.J., Van der Wal, J.E., Roodenburg, J.L. and Sterenborg, H.J. (2005). Autofluorescence and Raman microspectroscopy of tissue sections of oral lesions. *Lasers. Med. Sci.,* 19: 203-209.

59. Warnakulasuriya, S., Johnson, N.W. and van der Waal, I. (2007). Nomenclature and classification of potentially malignant disorders of the oral mucosa. *J. Oral. Pathol. Med.,* 36: 575-580.

60. Warnakulasuriya, S., Reibel, J., Bouquot, J. and Dabelsteen, E. (2008). Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. *J. Oral. Pathol. Med.,* 37: 127-133.

61. Cals, F. L. J., Tom, B. C. S., Koljenovic, S., Puppels, G. J. and Baatenburg de Jong, R. J. (2013). Method development: Raman spectroscopy-based histopathology of oral mucosa. *J. Raman. Spectrosc.,* 44: 963-972.

62. Bergholt, M.S., Lin, K., Zheng, W., Lau, D.P. and Huang, Z. (2012). In vivo, real-time, transnasal, image-guided Raman endoscopy: defining spectral properties in the nasopharynx and larynx. *J. Biomed. Opt.,*17: 077002.

63. Smits, R.W., Koljenovic, S., Hardillo, J.A., Ten Hove, I., Meeuwis, C.A., Sewnaik, A., Dronkers, E.A., Bakker Schut, T.C., Langeveld, T.P., Molenaar, J., Heqt, V. N., Puppels, G. J. and Baatenburg de Jong, R. J. (2015). Resection margins in oral cancer surgery: Room for improvement. *Head. neck.,*

64. Wu, J.G., Xu, Y.Z., Sun, C.W., Soloway, R.D., Xu, D.F., Wu, Q.G., Sun, K.H., Weng, S.F. and Xu, G.X. (2001). Distinguishing malignant from normal oral tissues using FTIR fiber-optic techniques. *Biopolymers.,* 62: 185-192.

65. Wen-Liang Lo, W. L., Lai, J. Y., Feinberg, S. E., Izumi, K., Kao, S. Y., Chang, C. S., Lin, A. and Chiang, H. K. (2011). Raman spectroscopy monitoring of the cellular activities of a tissue-engineered ex vivo produced oral mucosal equivalent. *J. Raman. Spectrosc.,* 42: 174-178.

66. Bergholt, M. S. and Huang, W. Z. (2012). Characterizing variability in in vivo Raman spectroscopic properties of different anatomical sites of normal tissue in the oral cavity. *J. Raman. Spectrosc.,* 43: 255-262.

67. Daniel, A., Aruna, P., David, B., Leena, J., Krishna, M. C., Koteeswaran, D. and Ganesan, S. (2014). Raman mapping of oral tissues for cancer diagnosis. *J. Raman. Spectrosc.,* 45: 541-549.

68. Huang, X., Ivan, H., El-Sayed., Mostafa, A. and El-Sayed. (2010). Applications of gold nanorods for cancer imaging and photothermal therapy. *Cancer. Nanotechnol.,* 624: 343-357.

69. Forastiere, A., Koch, W., Trotti, A. and Sidransky, D. (2001). Head and neck cancer. *N. Engl. J. Med.,* 345: 1890-1900.

70. Vokes, E.E., Weichselbaum, R.R., Lippman, S.M. and Hong, W.K. (1993). Head and neck cancer. *N. Engl. J. Med.,* 328: 184-194.

71. Siegel, M.A., Kahn, M.A. and Palazzolo, M.J. (2009). Oral cancer: a prosthodontic diagnosis. *J. Prosthodont.,* 18: 3-10.

72. Farah, C.S. and McCullough, M.J. (2008). Oral cancer awareness for the general practitioner: new approaches to patient care. *Aust. Dent. J.,* 53: 2-10.

73. Valdés, R., Stefanov, S., Chiussi, S., López-Alvarez, M. and González, P. (2014). Pilot research on the evaluation and detection of head and neck squamous cell carcinoma by Raman spectroscopy. *J. Raman. Spectrosc.,* 45: 550-557.

74. Slaughter, D.P., Southwick, H.W. and Smejkal, W. (1953). Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer.,* 6: 963-968.

75. Coleman, M.P., Gatta, G., Verdecchia, A., Esteve, J., Sant, M., Storm, H., Allemani, C., Ciccolallo, L., Santaquilani, M. and Berrino, F. (2003). EUROCARE-3 summary: cancer survival in Europe at the end of the 20th century. *Ann. Oncol.,*14: 128-149.

76. Neville, B.W. and Day, T.A. (2002). Oral cancer and precancerous lesions. *CA. Cancer. J. Clin.,* 52: 195-215.

77. Campo-Trapero, J., Cano-Sanchez, J., Palacios-Sanchez, B., Sanchez-Gutierrez, J.J., Gonzalez-Moles, M.A. and Bascones-Martinez, A. (2008). Update on molecular pathology in oral cancer and precancer. *Anticancer. Res.,* 28: 1197-1205.

78. Behl, I., Kukreja, L., Deshmukh, A., Singh, S.P., Mamgain, H., Hole, A.R. and Krishna, C.M. (2014). Raman mapping of oral buccal mucosa: a spectral histopathology approach. *J. Biomed. Opt.,* 19: 126005.

79. Karabulut, A., Reibel, J., Therkildsen, M.H., Praetorius, F., Nielsen, H.W. and Dabelsteen, E. (1995). Observer variability in the histologic assessment of oral premalignant lesions. *J. Oral. Pathol. Med.,* 24: 198-200.

80. Abbey, L.M., Kaugars, G.E., Gunsolley, J.C., Burns, J.C., Page, D.G., Svirsky, J.A., Eisenberg, E., Krutchkoff, D.J. and Cushing, M. (1995). Intraexaminer and interexaminer reliability in the diagnosis of oral epithelial dysplasia. *Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod.,* 80: 188-191.

81. Bigio, I.J. and Bown, S.G. (2004). Spectroscopic sensing of cancer and cancer therapy: current status of translational research. *Cancer. Biol. Ther.,* 3: 259-267.

82. Driemel, O., Kunkel, M., Hullmann, M., von Eggeling, F., Muller-Richter, U., Kosmehl, H. and Reichert, T.E. (2007). Diagnosis of oral squamous cell carcinoma and its precursor lesions. *J. Dtsch. Dermatol. Ges.,* 5: 1095-1100.

83. Glassman, A.B. (1998). Cytogenetics, in situ hybridization and molecular approaches in the diagnosis of cancer. *Ann. Clin. Lab. Sci.,* 28: 324-330.

84. Grassel-Pietrusky, R., Deinlein, E. and Hornstein, O.P. (1982). DNA-ploidy rates in oral leukoplakias determined by flow-cytometry. *J. Oral. Pathol.,*11: 434-438.

85. Vaidya, M.M., Borges, A.M., Pradhan, S.A., Rajpal, R.M. and Bhisey, A.N. (1989). Altered keratin expression in buccal mucosal squamous cell carcinoma. *J. Oral. Pathol. Med.,* 18: 282-286.

86. Sokolov, K., Follen, M. and Richards-Kortum, R. (2002). Optical spectroscopy for detection of neoplasia. *Curr. Opin. Chem. Biol.,* 6: 651-658.

87. Wilder-Smith, P., Holtzman, J., Epstein, J. and Le, A. (2010). Optical diagnostics in the oral cavity: an overview. *Oral. Dis.,* 16: 717-728.

88. Caspers, P. J., Lucassen, G. W., Carter, E. A., Bruining, H. A. and Puppels, G. J. (2002). invivo confocal Raman microspectroscopy of the skin: Noninvasive determination of molecular concentration profiles. *J. Invest. Dermatol.,* 116: 434-442.

89. Crow, P., Stone, N., Kendall, C.A., Persad, R.A. and Wright, M.P. (2003). Optical diagnostics in urology: current applications and future prospects. *BJU. Int.,* 92: 400-407.

90. Wong Kee Song, L.M. and Wilson, B.C. (2005). Endoscopic detection of early upper GI cancers. *Best. Pract. Res. Clin. Gastroenterol.,* 19: 833-856.

91. Pujary P.K., M., C., Krishna, M. and Pujary, K. (2011). Raman Spectroscopic Methods for Classification of Normal and Malignant Hypopharyngeal Tissues: An Exploratory Study. *Patholog Res Int.,* Article ID 632493: 1-9.

92. Rehman, S., Movasaghi, Z., Tucker, A.T., Joel,P.S., Darr,A.J., Ruban,V.A. and Rehman, UR. I. (2007). Raman spectroscopic analysis of breast cancer tissues: identifying differences between normal, invasive ductal carcinoma and ductal carcinoma in situ of the breast tissue. *J. Raman. Spectrosc.,* 38: 1345-1351.

93. Movasaghi, Z., Shazza, R. and Rehman, I.U. . (2012). Raman Spectroscopy Can Detect and Monitor Cancer at Cellular Level: Analysis of Resistant and Sensitive Subtypes of Testicular Cancer Cell Lines. *Appl. Spectros. Rev.,* 47: 571-581.

94. Liu, F.T., Agrawal, S.G., Movasaghi, Z., Wyatt, P.B., Rehman, I.U., Gribben, J.G., Newland, A.C. and Jia, L. (2008). Dietary flavonoids inhibit the anticancer effects of the proteasome inhibitor bortezomib. *Blood.,* 112: 3835-3846.

95. K. Venkatakrishna, Kurian, J., Pai, K. M., Krishna, C. M., Ullas, G. and Kartha, V. B. (2001). Optical pathology of oral tissue: a Raman spectroscopy diagnostic method. *Curr. Sci.,* 80: 101-5

96. Krishna, C.M., Sockalingum, G.D., Kurien, J., Rao, L., Venteo, L., Pluot, M., Manfait, M. and Kartha, V.B. (2004). Micro-Raman spectroscopy for optical pathology of oral squamous cell carcinoma. *Appl. Spectrosc.,* 58: 1128-1135.

97. Guze, K., Short, M., Zeng, H., Lerman, M. and Sonis, S. (2011). Comparison of molecular images as defined by Raman spectra between normal mucosa and squamous cell carcinoma in the oral cavity. *J. Raman. Spectrosc.,* 42: 1232-1239.

98. Devpura, S., Thakur, J. S., Sethi, S., Naik, V. M. and Naik, R. (2012). Diagnosis of head and neck squamous cell carcinoma using Raman spectroscopy: tongue tissues. *J. Raman. Spectrosc.,* 43: 490-496.

99. Mian, SA., Colley, HE., Thornhill, MH., and Rehman, IU.; (2014) Development of a Dewaxing Protocol for Tissue-Engineered Models of the Oral Mucosa Used for Raman Spectroscopic Analysis; Applied Spectroscopy Reviews; Volume 49, Issue 8, pages 614-617.

100. Lloyd, G.R., Orr, L.E., Christie-Brown, J., McCarthy, K., Rose, S., Thomas, M. and Stone, N. (2013). Discrimination between benign, primary and secondary malignancies in lymph nodes from the head and neck utilising Raman spectroscopy and multivariate analysis. *Analyst.,* 138: 3900-3908.

101. Knipfer, C., Motz, J., Adler, W., Brunner, K., Gebrekidan, M.T., Hankel, R., Agaimy, A., Will, S., Braeuer, A., Neukam, F.W. and Stelzel, F. (2015). Erratum: Raman difference spectroscopy: a non-invasive method for identification of oral squamous cell carcinoma: publisher's note. *Biomed. Opt. Express.,* 6: 2675.

102. Singh, S. P. and Krishna, M. C. (2014). Raman spectroscopic studies of oral cancers: correlation of spectral and biochemical markers. *Anal. Methods.,* 6: 8613-8620.

103. Singh, S.P., Sahu, A., Deshmukh, A., Chaturvedi, P. and Krishna, C.M. (2013). In vivo Raman spectroscopy of oral buccal mucosa: a study on malignancy associated changes (MAC)/cancer field effects (CFE). *Analyst.,* 138: 4175-4182.

104. Shyam Sunder, N., Nirmala, N. R., Kartha, V. B., Ullas, G., Kurien, J. (2011) Laser Raman spectroscopy: a novel diagnostic tool for oral cancer. *J. Orofac. Sci.* 3: 15–19.

105. Singh, S.P., Deshmukh, A., Chaturvedi, P. and Krishna, M. C. (2012). In vivo Raman spectroscopic identification of premalignant lesions in oral buccal mucosa. *J. Biomed. Opt.,* 17: 105002

106. Lau, D. P., Haing, H., Lui, C. S., Man, K., Berean, M. D., Morrison, M. D. and Zeng H. (2003). Raman spectroscopy for optical diagnosis in normal and cancerous tissue of the nasopharynx - preliminary findings. *Laser. Sur. Med.,* 32: 210-214.

107. Feng, S., Lin, J., Cheng, M., Li, Y.Z., Chen, G., Huang, Z., Yu, Y., Chen, R. and Zeng, H. (2009). Gold nanoparticle based surface-enhanced Raman scattering spectroscopy of cancerous and normal nasopharyngeal tissues under near-infrared laser excitation. *Appl. Spectrosc.,* 63: 1089-1094.

108. Diem, M., Mazur, A., Lenau, K., Schubert, J., Bird, B., Miljkovic, M., Krafft, C. and Popp, J. (2013). Molecular pathology via IR and Raman spectral imaging. *J. Biophotonics.,* 6: 855-886.

109. Harris, A.T., Lungari, A., Needham, C.J., Smith, S.L., Lones, M.A., Fisher, S.E., Yang, X.B., Cooper, N., Kirkham, J., Smith, D.A., Martin-Hirsch, D. P. and High, A. S. (2009). Potential for Raman spectroscopy to provide cancer screening using a peripheral blood sample. *Head. Neck. Oncol.,* 1: 34.

110. Sahu, A., Sawant, S., Mamgain, H. and Krishna, C.M. (2013). Raman spectroscopy of serum: an exploratory study for detection of oral cancers. *Analyst.,* 138: 4161-4174.

111. Rekha, P., Aruna, P., Bharanidharan, G., Koteeswaran, D., Baludavid, M. and Ganesan, S. (2015). Near infrared Raman spectroscopic characterization of blood plasma of normal, oral premalignant and malignant conditions-a pilot study. *J. Raman. Spectrosc.,* 46: 735-743.

112. Scully, C., Bagan, J.V., Hopper, C. and Epstein, J.B. (2008). Oral cancer: current and future diagnostic techniques. *Am. J. Dent.,* 21: 199-209.

113. Qiu, S., Xu, Y., Huang, L., Zheng, W., Huang, C., Huang, S. and Pan, J. (2016). Non-invasive detection of nasopharyngeal carcinoma using saliva surface-enhanced Raman spectroscopy. *Oncol. Lett.,* 11: 884-890.

114. Kho, K. W., Olivo, M., Shen, Z. X. and Soo, K. C. (2005). Surface enhanced Raman spectroscopic (SERS) study of saliva in the early detection of oral cancer. In *Proceedings of SPIE*. pp 84-91.

115. Olivo, M., Bhuvaneswari, R. and Keogh, I. (2011). Advances in bio-optical imaging for the diagnosis of early oral cancer. *Pharmaceutics.,* 3: 354-378.

116. Yan, B., Li,B., Wen, Z., Luo, X., Xue, L. and Li, L. (2015). Label-free blood serum detection by using surface-enhanced Raman spectroscopy and support vector machine for the preoperative diagnosis of parotid gland tumors. *BMC. Cancer.,* 15.

117. Kinoshita, H., Miyoshi, N., Miyoshi, K., Ogawa, T., Ogasawara, T., Kitagawa, Y., Itoh, H. and Sano, K. (2008). Phosphate and amide III mapping in sialoliths with Raman microspectroscopy. *J. Raman. Spectrosc.,* 39: 349-353.

118. Schrotzlmair, F., Muller, M., Pongratz, T., Eder, M., Johnson, T., Vogeser, M., von Holzschuher, V., Zengel, P. and Sroka, R. (2015). Laser lithotripsy of salivary stones: Correlation with physical and radiological parameters. *Lasers. Surg. Med.,*47: 342-349.

119. Bergholt, M.S., Zheng, W., Lin, K., Ho, K.Y., Teh, M., Yeoh, K.G., So, J.B. and Huang, Z. (2011). Characterizing variability in in vivo Raman spectra of different anatomical locations in the upper gastrointestinal tract toward cancer detection; *J. Biomed. Opt.* 16: 255-262.

Table 1: Raman peaks and the characteristic assignments related to soft tissue analysis

Raman shift (cm^{-1})	**Peak assignment**	**References**
2925-2959	C-H stretching band for normal tissues	64
1740-1745	C = O vibration of triglycerides for normal tissues	64
1698--1699	Amide I	116
1662	Nucleic acid	113
1655	Amide I	65
1619	Tryptophan	113
1607	C = C band in Phenylalanine or Tyrosine	116
1583	Cell surface protein spectral biomarker	97
1541--1551	C-N stretching, Amide II	116
1450-1660	Protein	102
1448	CH_2 bending mode of collagen	113
1441--1445	CH_2, CH_3 bending in proteins and lipids	116, 102
1440	Lipid	102
1541--1551,1441--1445	Guanine in DNA, Tryptophan in proteins	116

1368--1373, 1607		
1336	Nucleic acid	113
1326--1329	CH vibration in DNA/RNA, CH_2 twisting in lipids	116
1270	C-C waver mode of fatty acids	113
1265	Amide III	118
1261--1264	CH bending in lipids	116
1254	Amide III	65
1200-1350	Protein compliance and C-H bond in nucleic acid bases	56
1140	Carotenoids	116
1134	Glycogen	113
1127	C-C stretching in lipids, C-N stretching in D-Mannos	116
1094	C-N stretching mode in D-Mannos	116
1084	C-C stretching mode in phospholipids	116
1004	Phenylalanine	65
1003	Phenylalanine	113

960	Phosphate	118
933	C-C stretching mode, C-C αhelix in proteins	116
812	L-Serine	113
744--747	Thymine in DNA	116
729	C-H in plane bending mode of adenine	113
723--727	Hypoxanthine	116
635	Tyrosine	113
543--548	S-S disulfide stretching in Proteins	116
496	D-mannose	113
292--296	Au-S band	116

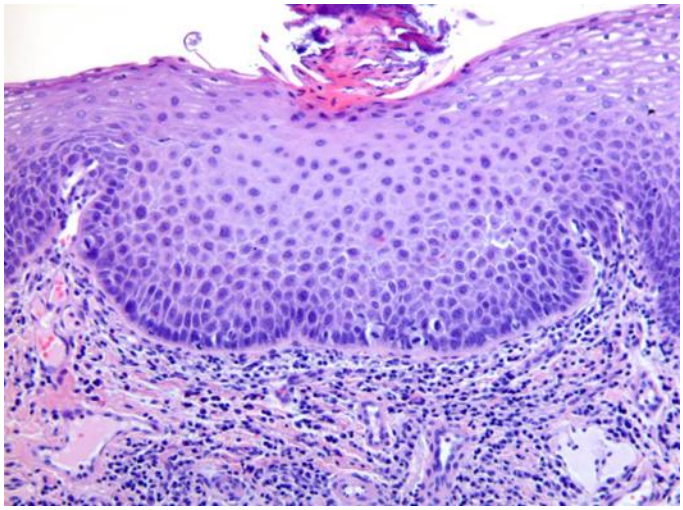

Figure 1(a); Photomicrograph showing area of increased epithelial thickness together with hyperkeratosis: mild dysplasia (WHO) or parabasal hyperplasia (Ljubljana) [Reproduced with permission] [60]

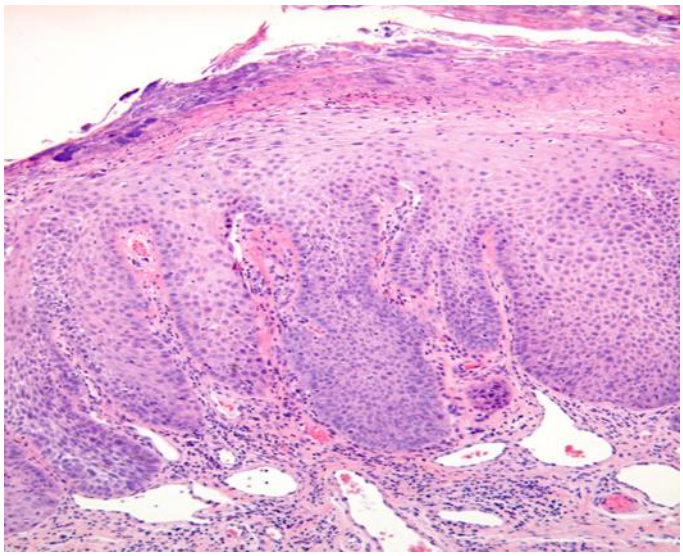

Figure 1b; Photomicrograph showing blunt and elongated epithelial ridges and cytonuclear atypia confined to the lower epithelial half: moderate dysplasia (WHO) or atypical hyperplasia (Ljubljana). [Reproduced with permission] [60]

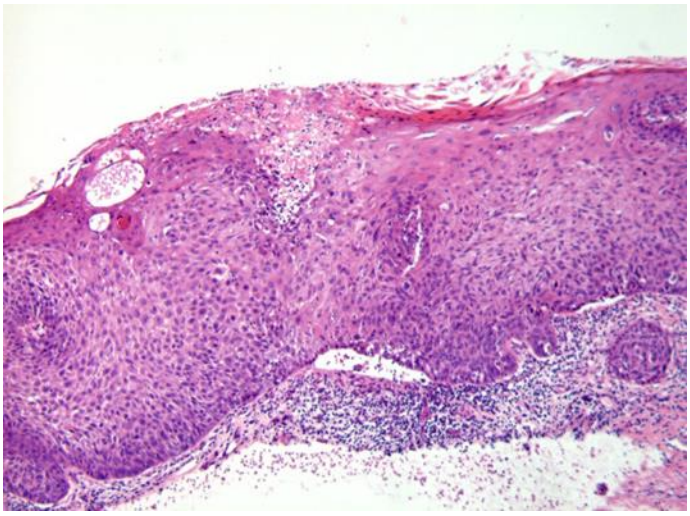

Figure 1c; Photomicrograph showing epithelial alterations involving the entire epithelial thickness: severe dysplasia (WHO) or atypical hyperplasia (Ljubljana). [Reproduced with permission] [60]

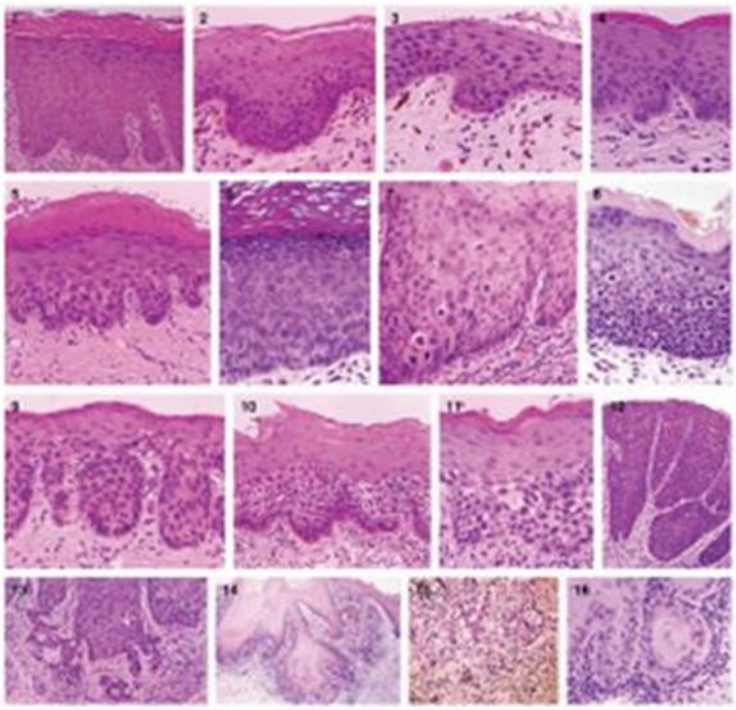

Figure 1d; Oral epithelial dysplasia classification system: predictive value, utility, weaknesses and scope for improvement (WHO) (Ljubljana) [Reproduced with permission] [60]

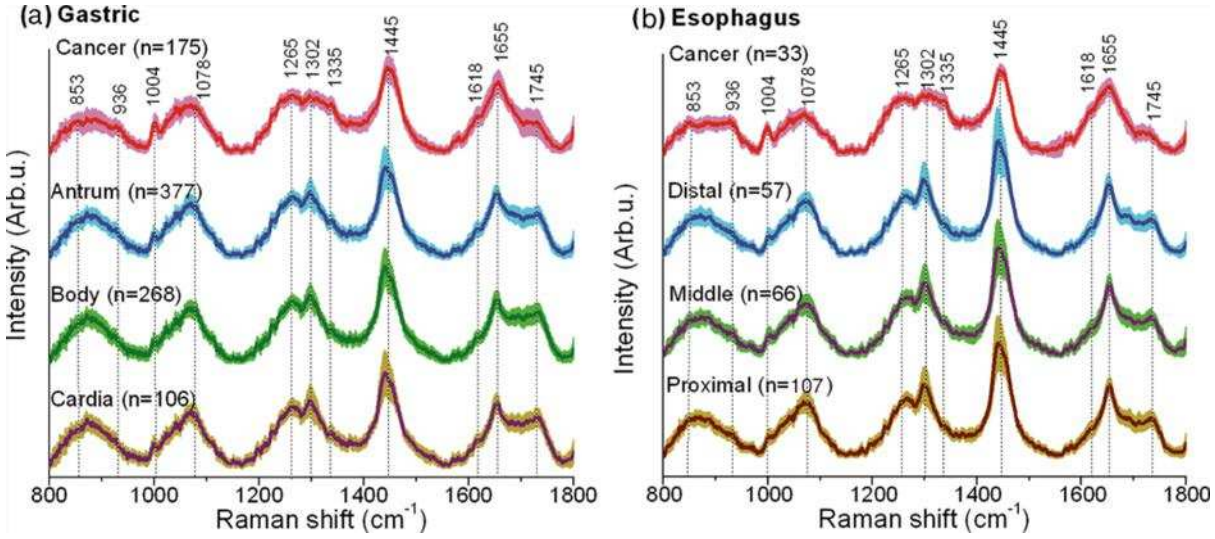

Figure 2; (a) In vivo mean Raman spectra ±1 SD of normal (antrum, body, and cardia) and neoplastic gastric tissues. (b) In vivo mean Raman spectra ±1 SD of the normal (distal, middle, and proximal) and neoplastic esophageal tissues. Note that the mean Raman spectra are vertically shifted for better visualization. [Reproduced with permission from SPIE, **J. Biomed. Opt. 2011; 16(3):037003-037003-10. doi:10.1117/1.3556723**] (119)

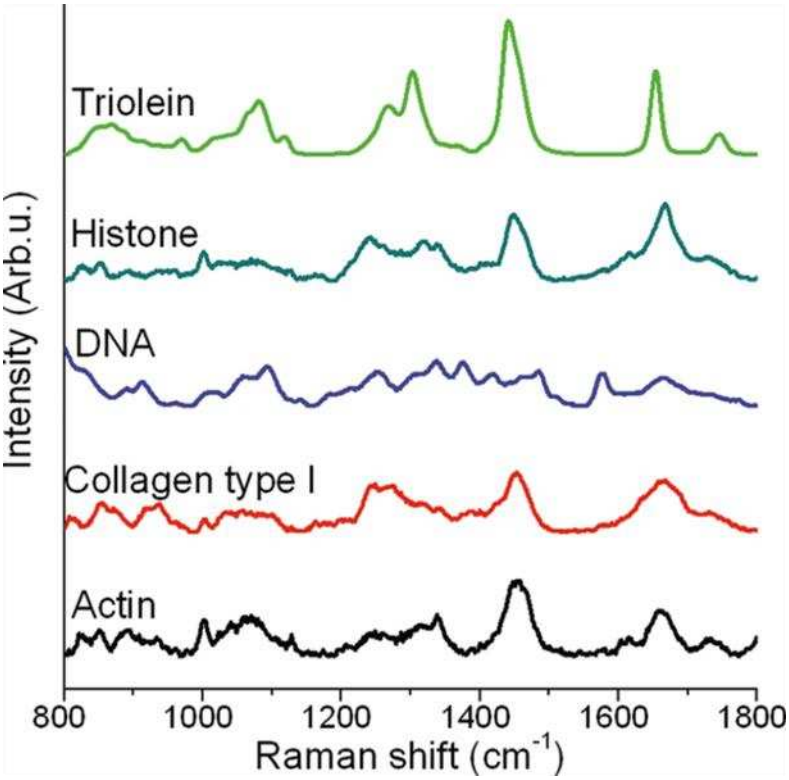

Figure 3: The five basis reference Raman spectra (i.e., actin, collagen, DNA, histones, and triolein) are used for biochemical modeling of *esophageal and* gastric tissue. [Reproduced with permission from SPIE, **J. Biomed. Opt. 2011; 16(3):037003-037003-10. doi:10.1117/1.3556723**] (119)

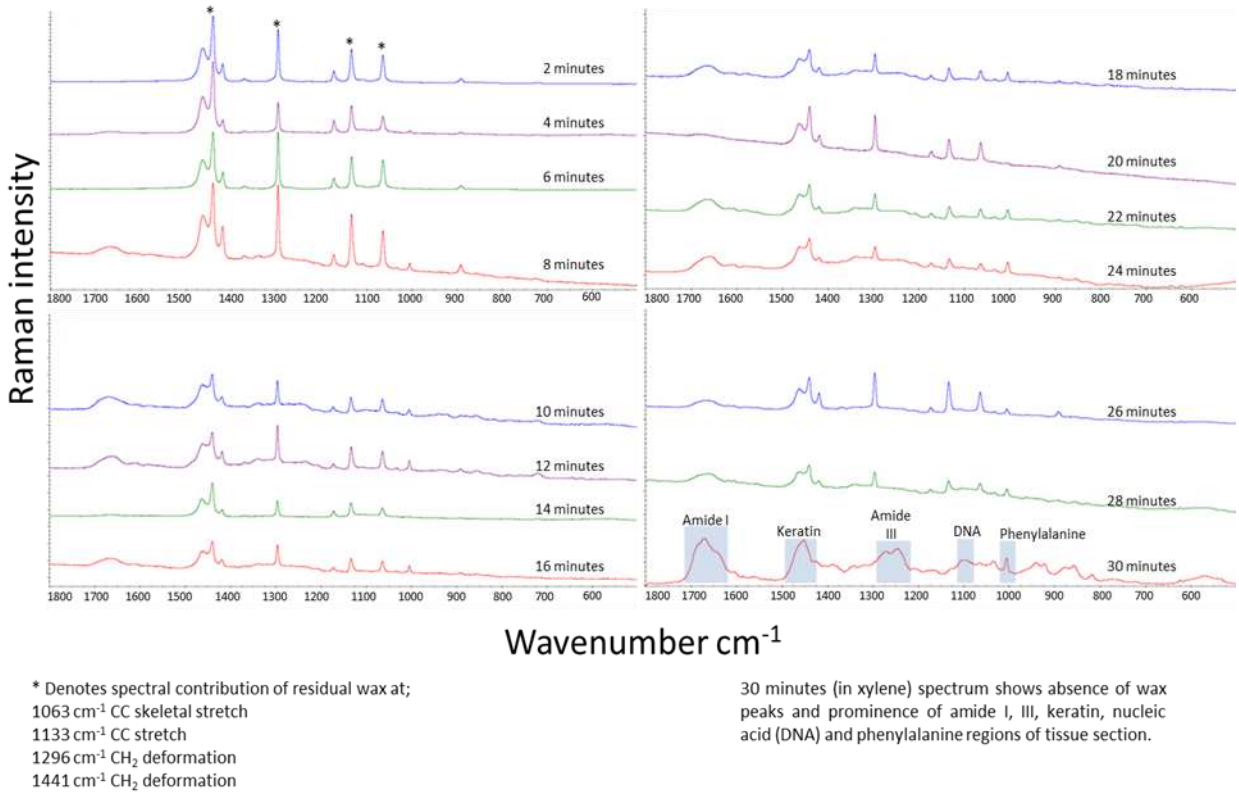

Figure 4; Raman spectra of 20 µm tissue sections after de-waxing in xylene for increasing lengths of time (2 to 30 minutes). (99)