Autofluorescence Spectroscopy of Oral Submucous Fibrosis

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CERTIFICATE

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INTRODUCTION

It is approximately half a century since Schwartz described oral submucous fibrosis in the tobacco-chewing women of Indian origin in Kenya. Since then this condition evoked an intense enthusiasm among many researchers in India and through out the world. Various authors had investigated the condition thoroughly and proposed several factors that play a role in the etiopathogenesis of this condition. Current evidence suggests that arecoline in the areca nut is the key factor in initiating the disease process.

This condition is aptly described by Pindborg and Sirsat as "an insidious chronic disease affecting any part of the oral cavity and some times the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat".

The habit of betel quid chewing is widespread throughout India and South East Asia. This condition is also reported in Asian immigrants living in other parts of the world. Various researchers have conducted studies in different parts of the country to check out the incidence of betel quid chewing habit in general population. At present this habit is widely prevalent in teenagers and young adults. In one study it is found that the relative incidence of this habit is approximately around 5% in the general population. In another report it is stated that 0.4% of Indian villagers has the habit of betel quid or pan chewing.

Oral submucous fibrosis predominantly involves the oral cavity. The buccal mucosa, retromolar area, and the soft palate are the predominantly affected sites. The mucosa in the involved areas gradually becomes pale followed by progressive stiffness of subepithelial tissues. In addition to the involvement of oral mucosa this condition also involves the pharynx and esophagus in persons who chew and swallow the products of betel quid. Recently, an increased incidence of malignancy is noted in oral submucous fibrosis patients, particularly in people who use commercially available products. The malignant transformation of betel quid users in India is around 8%, which is quite high.

The most ironical aspect of this condition is lack of appropriate treatment modalities. Unlike tobacco pouch keratosis, oral submucous fibrosis does not regress with the habit cessation, although mild cases may be treated with intralesional corticosteroids to reduce the symptoms. Surgical splitting and excision of the fibrous bands have also been tried to improve the mouth opening in later stages of the disease. A recent study showed that intralesional injections of interferon gamma improved maximum mouth opening, reduced mucosal burning and increased suppleness of the buccal mucosa.

Since the habit of betel quid chewing and other commercially available products is wide spread in our country we need techniques that can be helpful for mass screening of the public to identify this condition at an earlier stage. Autofluorescence is one such technique that can be used for mass screening. This technique of autofluorescence has been used with considerable success in identifying various carcinomas including oral squamous carcinoma.

Autofluorescence spectroscopy is a non-invasive and easily applicable tool for the detection of alterations in the structural and chemical compositions of cells, which may indicate the presence of diseased tissue. Autofluorescence of tissues is due to several endogenous fluorophores. These comprise fluorophores from tissue matrix molecules and intracellular molecules like tryptophan, collagen, elastin, and NADH. Early detection of pre-malignant lesions, premalignant conditions and malignant tumors may reduce patient morbidity and mortality, and therefore is of great clinical importance.

Several authors used this technique of autofluorescence to differentiate various precancerous and cancerous conditions from normal mucosa using both in vitro and in vivo methods. The intensities of these fluorophores are altered in various precancerous conditions (submucous fibrosis), precancerous lesions (leukoplakia and erythroplakia) and in carcinomas of oral cavity.

In the present study we are using the technique of autofluorescence to detect the mucosal and submucosal changes of normal controls, betel quid chewers and oral submucous fibrosis individuals.

REVIEW OF LITERATURE

HISTORICAL REVIEW

1. This condition was first described in ancient Indian Medical Manuscripts by Sushruta at the time of around 400 B.E. describing it as “VEDARI” where he gives description of patients suffering from narrowing of mouth, burning sensation and pain. (ABROL 1977)[1].

2. Schwartz (1952)[1] reported a condition consisting of limitation of mouth opening amongst south Indian women in Kenya, which he named "ATROPHIA IDIOPATHICA (TROPICA) MUCOSA ORIS".
3. Joshi (1953)[1] an ENT surgeon observed this condition and termed as "SUBMUCOUS FIBROSIS OF PALATE AND PILLARS OF FAUCES".
4. Since then several authors coined various terminologies for this condition. These terminologies are summarized by Abrol[1] which are,

LAL (1953) - Diffuse oral submucous Fibrosis

SU (1954) – Idiopathic scleroderma of mouth

DESA (1957) – Submucous fibrosis of palate and cheek

GEORGE (1958) - Submucous fibrosis of palate and mucous membrane.

RAO (1962) - Idiopathic palatal fibrosis

BEHL (1962) – Sclerosing stomatitis

PINDBORG and SIRSAT (1964) – Oral submucous fibrosis.

GOLERIA (1970) - Subepithelial fibrosis

ABROL et al (1972) - Idiopathic oral fibrosis.

Of all the terminologies in the literature the term "ORAL SUBMUCOUS FIBROSIS" is currently widely used.

ETIOLOGY

Although various etiological agents are proposed, the exact etiology of oral submucous fibrosis has not yet been identified. Current evidence suggests that arecoline in areca nut[39] plays a major role in initiating the

disease process. Various etiological agents are summarized by Abrol[1], which are,

1. **Su** (1954) attributed it to the tannic acid and arecoline contents of betel nut, together with the influence of lime.
2. **Rao** (1962) linked it to collagenopathies.
3. **Sirsat** and **Khanolkar** (1962) attributed it to irritation caused by capsaicin. (Gupta. D.S. et al 1980)
4. **Pindborg** and **Sirsat** (1964) postulated it as a collagen disease caused by irritation from chillies and hot spicy foods.

5. **Abrol** and **Krishnamoorthy** (1970) suggested a genetic predisposition with supra added local irritation from betel nut, chillies, spices and condiments.
6. **Abrol** and **Raveendran** (1972) stated it as a precancerous condition due to recurrent chemical irritation and inflammation of oral mucosa caused by lime, spices, condiments and tobacco coupled with a genetic predisposition.
7. **Sirsat** and **Khanolkar** proved the occurrence of fibrosis by applying capsaicin to rat palatal mucosa but could not prove it by applying arecoline which is the most active ingredient in betel nut. (Gupta D.S. et al 1980).
8. **Ramanathan. K** (1981)[58] is of the view that SMF is an Asian version of Sideropenic dysphagia. He suggests that SMF appears to be an altered oral mucosa following a prolonged deficiency of iron and/or vitamin B complex, especially folic acid. This altered oral mucosa subsequently develops hypersensitivity to oral irritants such as spices especially chillies and betel quid.

9. **Canif. J.P** and **Harvey. W** (1986)[14] proposed two etiological factors in the development of OSMF, which are, genetic predisposition and betel nut chewing. They propose that HLA antigens A10, DRJ, DR7 together with autoantibodies constitute an autoimmune basis for OSMF. They also stated that an alkaloid in areca nut stimulates collagen synthesis and proliferation of buccal mucosal fibroblasts. Tannins that is present in betel nut increase the resistance of collagen to degradation which further enhances fibrosis.
10. **Sinor P.N**. et al (1990)[61] suggested that areca nut is the most probable causative agent. He postulated that chewing of mawa (90% Areca nut) along with tobacco enhanced the risk of SMF. His study revealed that areca nut chewing results in early onset of disease and fibrous bands in posterior part of oral cavity, while chewing of areca nut along with tobacco, betel leaves and lime resulted in late onset of disease with fibrous bands in anterior parts of oral cavity.
11. **Binnie** and **Cawson** (1972)[8] were the first to reveal the muscle involvement in OSMF that was further supported by Caniff and Pillai.
12. **Khanna. J. N** and **Andrade. N. N** (1995)[39] depicted the role of areca nut in the pathogenesis of OSMF by a schematic representation.

MALIGNANT POTENTIAL

1. **Paymaster** first reported the development of slow growing oral carcinoma in 1/3rd of cases seen at Tata Memorial Hospital, Bombay (J.J. Pindborg et al, 1966).
2. **Pindborg** et al (1975)[55] reported an association between oral cancer and SMF among 100 patients with oral cancer in South India. He

found 40 patients suffering simultaneously from submucous fibrosis. Biopsies from 30 of 40 patients demonstrated epithelial atypia in 11.5% of areas of OSMF remote from the cancer. When biopsies were taken from areas of SMF in the vicinity of the cancer, epithelial atypia was 71.4%.

3. **Caniff. J.P** et al (1986)[14] studied 30 cases and he reported 8 cases (27%) with mild atypia 2 cases (7%) with moderate atypia and 1 case (3%) had marked atypia, which later turned into squamous cell carcinoma, two years later.
4. **Pindborg** and **Murthy**, after 15 years of follow-up reported a malignant potential of 4.5%.
5. **Shiau** and **Kwan** reported that the incidence of squamous cell carcinoma in OSMF is 25%. (Glenn Muraw et al 1987)[30].
6. **Maher. R**. et al (1996)[49] reported mild dysplasia in 28.3% (21 cases), moderate dysplasia in 9.5% (7 cases) out of 74 cases of SMF he studied.

CLINICAL FEATURES

1. Age incidences given by various authors, based on their studies varied between 10 to 60 years.
2. Sex incidence also varies amongst various studies, most authors suggested a male preponderance, but **Maher** (1996)[48] have given an increased female predilection.

3. The sites commonly involved according to **Wahi** et al (1966)[75], are palate (51.3%), Buccal Mucosa (44.2%) Tongue (2.7%) Lip and gingiva (0.9%).
4. **Pindburg** et al (1966)[53] **Gupta** et al (1980)[33] **Caniff** and **El-Labban** (1985)[13] reported involvement of pterygomandibular raphe, pillar of fauces and uvula in the later stages.
5. **Vaish** et al (1981)[73] suggested that the order of preponderance from maximum to minimum is buccal, labial, commissural lingual and palatal mucosa.
6. **Wahi** et al (1966)[75] classified submucous fibrosis into three clinical groups.

Group I – No symptoms referable to mucosal involvement, affects one or more commonly involved anatomic site, focal in character, shows pallor or whitish coloration, wrinkling of mucosa and minimal induration.

Group II – symptoms of soreness of mucosa or increased sensitivity to chillies. The lesion is diffuse, white, extensive, indurated involving one or more anatomical sites.

Group III – Symptoms mainly due to restricted mouth opening, stretching of angles of mouth, inability to protrude the tongue, presence of altered pronunciation and palpable firm submucosal bands.

7. **Pindborg** et al (1966)[53] **Gupta** et al (1980)[33] **Glenn Morawetz** et al (1987)[51] postulated that the earliest symptoms include burning sensation, intolerance to hot spicy foods, blistering of oral mucosa with ulcerations and recurrent vesicles which are common on buccal mucosa, anterior faucial pillars, soft palate and labial mucosa.
8. **Caniff. J.P** et al (1986)[14] reported a relative loss of auditory function due to stenosis of the opening of the Eustachian tube.

9. **Borle. R.M** and **Borle. S.M** (1991)[10] have classified oral submucous fibrosis clinically into two phases,
   i. An eruptive phase, characterized by formation of vesicles, erythema, burning sensation. The vesicles rupture to form small ulcers, which leads to further increase in burning sensation.
   ii. Fibrosis induction phase characterized by disappearance of vesicles, healing of ulcers, decreased burning sensation, blanching and stiffness of oral and oropharyngeal mucosa and healing by fibrosis.

10. **Caniff. J.P** (1986)[14] have reported that as the disease progresses, mucous membrane develops a blanched appearance. Gradually the mucosa becomes thick and inelastic. Erythematous patches occur in the affected areas. Fibrosis of mucosa occurs followed by stiffness, most commonly in palate, buccal mucosa and faucial pillars. In early cases, fibrous tissue is seen arching from anterior pillars into soft palate. As the disease progresses, thick inextensible fibrous bands develop vertically along the cheeks. Floor of mouth becomes pale and thickened. In advanced cases jaws are inseparable, and a totally inelastic mucosa is forced against buccal aspects of teeth where sharp edges or restoration cause ulcerations which become secondarily infected. The fibrosis progresses into the posterior part of buccal mucosa, anterior pillar of fauces and soft palate. Uvula becomes small and distorted. On palpation of lower lip, circular band of fibrous tissue in felt over entire rima oris.
11. As the pterygomandibular raphe is fibrosed, the base of tongue that is attached to it is pulled back and patient complains of inability to protrude the tongue. Further atrophy of papillae, dysphasia and

xerostomia are also seen (**Caniff** et al, **Morawetz** et al, M. **McGurk** et al). Degree of trismus depends upon the degree of fibrosis and the area of mucosa involved.

12. Other features reported include referred pain in ear, deafness, nasal twang in speech (**Moos** and **Madan** 1968)[50], clinical evidence of leukoplakia (**Wahi** et al 1966)[75], (**Pindborg** 1966)[53], and occurrence of hyper-pigmented areas adjacent to areas of normal mucosa. (**Pindborg** et al 1980)[54].

HISTOPATHOLOGICAL FEATURES

1. **Joshi** (1953)[1] mentions frequent presence of intraepithelial vesicles in early stages of disease. Other changes include parakeratosis, signet cell degeneration, liquefaction degeneration of basal layers.
2. **Pindborg**. J.J, **Mehta F.S**, **Daftary. D.K** (1970)[55], out of 53 cases of biopsy specimens observed, 71.7% of biopsies showed atrophic epithelium, normal thickness in 26.4% and hyperplastic epithelium in 1.9%. In 26% of cases buccal mucosa showed hyperothokeratosis, 22% showed hyperparakeratosis and 52% showed unkeratinized surface. 22.6% cases showed epithelial atypia with intercellular edema. 19.2% of biopsies showed signet cells in basal layer. There was reduction of melanin pigment in basal cell layer and 3 biopsies revealed presence of colloid bodies in epithelium and marked lymphocyte infiltration in lamina propria.
3. **McGurk** et al (1984)[46] observed subepithelial chronic inflammatory reaction and accumulation of dense collagen at dermo-epidermal junction with extension of the fibrosis down into the submucous and voluntary muscle.

4. **El-Laban. N. G** and **Caniff. J.P.** (1985)[26] studied ultra structural findings of muscle degeneration in OSMF. He demonstrated severe necrosis in high proportion of muscle fibers.
5. **Caniff. J.P**, **Harvey**, **Harris** (1986)[14] examined 30 cases and showed atrophic epithelium in 26%, non keratinized epithelia in 33%, mild atypia 27% and moderate atypia 7%. All 30 cases (100%) showed collagen accumulation beneath basement membrane and chronic inflammatory cell infiltrate consisting of lymphocytes, plasma cells, monocytes, and macrophages within lamina propria.

6. **De Waal** et al (1997)[22] studied fibroblasts content in SMF. They observed an increase in F-3 cells which produced type I and type III collagen in excess amounts in oral submucous fibrosis.

IMMUNOLOGICAL, BIOCHEMICAL AND HAEMATOLOGIC FEATURES

1. **Pindborg** et al observed mild iron deficiency anaemia and mild neutropenia in 40% of their cases.
2. **Dinesh. S. Gupta** et al (1980)[32] showed increased levels of IgG, IgM, IgA immunoglobulins suggesting an autoimmune basis for OSMF. They also proposed that the severity of SMF was directly proportional to estimated levels of major immunoglobulin.
3. **Rajendran. R** et al (1986)[57] assessed cell mediated and humoral response in 50 cases with OSMF. The number of high affinity rosette forming cells (HARFC) was decreased and levels of IgA, IgD, IgE was increased in OSMF.

4. **Caniff. J.P. , Harvey, Harris** (1986)[14] showed a mean increase in serum IgG concentration. In 30 out of 44 cases examined, antibody was present in 17 cases. A genetic predisposition involving HLA antigens A10, DR3, DR7, haplotypic pairs A10/DR3, B8/DR3, A10/B8 has been demonstrated.
5. **Glenn Morawetz** et al (1987)[30] reported an increase in ESR levels in OSMF patients.
6. **Scutt A** et al (1987)[60] observed that treatment of reconstituted collagen fibrils and pieces of rat dermis with the crude extract, purified tannins or (+)-catechin from betel nut (Areca catechu) increases their resistance to both human and bacterial collagenases in a concentration-dependent manner. These tanning agents may stabilize collagen in vivo following damage to the oral epithelium, and promote the sub-epithelial fibrosis which occurs in betel nut chewers.
7. **Chaturvedi. V. N, Marathe. N.G.** (1988)[15] estimated serum globulin and serum immunoglobulin IgG, IgA, IgM in 18 OSMF cases. Serum globulin was markedly raised in grade II and grade III cases. Serum IgG levels were marked in grade III cases compared to grade I cases. Serum IgA was decreased in grade III and unchanged in grade I and II. Serum IgM level did not show any significant change in OSMF.
8. **Anuradha. C. D** and **Shyamala Devi. C. S** (1998)[2] reported increased levels of eosinophils, decrease in hemoglobin levels and decreased MCH, MCHC, MCV levels. There was a decrease in serum iron content, serum copper, zinc level and an increase in iron binding capacity.

9. **Haque. M.F** et al (1997)[35] in their histochemical study of 30 OSF tissue specimens showed increased levels of CD4 to Cd8 and suggested an ongoing cellular immune response leading to alteration in local tissue architecture.
10. **Kaur J** et al (1999)[38] investigated the alterations in the expression of RAR-beta and p53 in OSF lesions and determined their association with disease pathogenesis. They found an altered expression of either RAR-B or p53 in majority of OSF lesions and suggested that it might be associated with disease pathogenesis.
11. **Trivedy C** et al (1999)[69] proposed immunolocalization of lysyl oxidase (LO) as a marker of fibrogenesis in oral submucous fibrosis (OSF). Oral biopsies from 13 subjects with OSF, 6 with histologically confirmed squamous cell carcinoma (SCC) arising in OSF and 10 SCC nonrelated to OSF, were examined. Strong positive staining was observed in 7/13 OSF samples in the cytoplasmic processes of fibroblasts and extracellularly in the upper third of the lamina propria. Furthermore, LO was found to co-localize in the areas stained strongly for collagen and elastin by histochemical stains. Examination of SCC tissues showed localization of LO adjacent to invading epithelial islands as evidence of a stromal reaction both in carcinomas arising from OSF and in SCC from non-OSF cases. These findings suggest that up regulation of LO may be an important factor in the pathogenesis of OSF and in the early stromal reaction of oral cancer.
12. **Chiang CP** et al (2000)[19] examined the PCNA expression in the oral epithelia of oral submucous fibrosis (OSF), epithelial hyperkeratosis (EH) and epithelial dysplasia (ED) under long-term exposure to areca quid. They used mouse monoclonal antibody PC10 to investigate

PCNA expression in histologic sections of OSF, EH, ED and normal oral mucosa (NOM). Positive PCNA staining was found mainly in basal and parabasal epithelial cells in all specimens of OSF, EH, ED and NOM. No significant correlation was found between PCNA LI in OSF epithelium and the clinicohistologic parameters of OSF. In addition, the mean PCNA LI of p53-positive OSF cases (23.7+/-12.0%) was very close to that of p53-negative OSF cases (23.9+/-13.1%), suggesting that there was no association between PCNA and p53 expression in OSF.

13. **Mythily Srinivasan** et al (2001)[67] evaluated the expression of EGFR and its ligand TGF-α in oral leukoplakia (OL) with dysplasia and OSMF as intermediate markers of malignancy by quantitative immunohistochemistry. They concluded that EGFR and TGF-a represent early markers of malignancy in OL with dysplasia.

AUTOFLUORESCENCE

HISTORY OF FLUORESCENT PROBES

Emission of light by a matter (luminescence) has always been known to man. Lightning in the sky, light emission by bacteria in the sea or by decaying organic matter are common natural phenomena. Scientific investigation of the luminescence phenomena began when the Bolognian stone was discovered in 1603.

1603 - Vincenzo Casciarolo, a Bolognian shoemaker and an alchemist, prepared by accident an artificial phosphor known as the Bolognian stone (or Bolognian phosphor) which glows after exposure to light.

16?? - Galileo Galilei (1564-1642), an Italian scientist, had the

view on the Bolognian stone: "It must be explained how it happens that the light is conceived into the stone, and is given back after some time, as in childbirth."

1646- Athanasius Kircher, a German Jesuit priest, recorded an interesting observation of the wood extract of *Lignum nephriticum*. An aqueous infusion of this wood exhibited blue color by reflected light and yellow color by transmitted light. The blue light is actually a type of light emission (fluorescence) and therefore Kircher is often regarded as the discoverer of fluorescence.

1838- David Brewster, a Scottish preacher, used the term "internal dispersion" to describe fluorescence phenomena.

1852- George Stokes, professor of mathematics and physics at Cambridge, interpreted the light-emitting phenomenon and formulated the law (the Stokes Law or the Stokes Shift) that the fluorescent light is of longer wavelength than the exciting light.

1853- Stokes coined the term "fluorescence" from the term "internal dispersion."

1856- William Perkin, an English chemist, synthesized a coal-tar dye, aniline purple (the first synthetic dye). His breakthrough attracted the attention of numerous synthetic chemists and a variety of dyes were synthesized. Perkin was acknowledged as the founder of the synthetic dye industry.

1864- Stokes lectured "On the application of the optical properties to detection and discrimination of organic substances" before the Chemical Society and the Royal Institution.

1871- Adolph Von Baeyer, a German chemist, synthesized a fluorescent dye, fluoresceine.

1880- A German firm known as Dr. G. Greublers Chemisches Laboratorium started to test and package the most desirable dyes for biologists and medical researchers.

1882- Paul Erlich, a German bacteriologist, employed the fluorescent dye uranin (sodium salt fluorescein) to track the pathway of secretion of aqueous humor in the eye. *This is the first case of the use of* in vivo *fluorochroming in animal physiology*.

1887- Karl Noack, a professor in Geissen, published a book listing some 660 fluorescent compounds arranged according to the color of their fluorescent light.

1897- Richard Meyer, a German chemist, introduced the term "fluorophores" for chemical groups with which fluorescence was associated.

1908- In Heinrich Kayser´s "Handbuch der Spectroscopie, vol. 4" Heinrich Knoen, a German physicist, arranged 1700 fluorescent compounds alphabetically with references to literature.

1911, 1913 - The first fluorescence microscope was developed by O. Heimstaedt, a German physicist, (1911) and H. Lehmann, a German physicist, (1913) as an outgrowth of the UV microscope (1901-1904). The instrument was used to investigate the autofluorescence of bacteria, protozoa, plant and animal tissues, and bioorganic substances such as albumin, elastin, and keratin.

1914- S. Von Provazek, a German protozoologist, employed the fluorescence microscope to study dye binding to living cells. He stated that fluorochromes introduced into the cell effectively illuminate the partial functions of the cell in the dark field of the fluoresence microscope. *This was a giant step forward in experimental cytology*.

1929- Philipp Ellinger, a German pharmacologist, and August

Hirt, a German anatomist, modified the fluorescence microscope so that it could be used to examine opaque specimens from most living organs. The new instrument was called an "intravital microscope" and is considered as the first epi-fluoresence (or incident-light excitation) microscope.

FLUORESCENCE: THE PHENOMENON

Fluorescence is the phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light at longer wavelengths. The distribution of wavelength-dependent intensity that causes fluorescence is known as the fluorescence excitation spectrum, and the distribution of wavelength-dependent intensity of emitted energy is known as the fluorescence emission spectrum.

Fluorescence detection has three major advantages over other light-based investigation methods: high sensitivity, high speed, and safety. The point of safety refers to the fact that samples are not affected or destroyed in the process, and no hazardous byproducts are generated.

Sensitivity is an important issue because the fluorescence signal is proportional to the concentration of the substance being investigated. Relatively small changes in ion concentration in living cells can have significant physiological effects. Whereas absorbance measurements can reliably determine concentrations only as low as several tenths of a micromolar, fluorescence techniques can accurately measure concentrations one million times smaller -- pico- and even femtomolar. Quantities less than an attomole (<10-18 mole) may be detected.

Using fluorescence, one can monitor very rapid changes in concentration. Changes in fluorescence intensity on the order of picoseconds can be detected if necessary.

Because it is a non-invasive technique, fluorescence does not interfere with a sample. The excitation light levels required to generate a fluorescence signal are low, reducing the effects of photo-bleaching, and living tissue can be investigated with no adverse effects on its natural physiological behavior.

FLUORESCENCE LIFETIME APPLICATIONS

In the last twenty years, fluorescence spectroscopy has evolved into a powerful tool for the study of chemical, semiconductor, photochemical, and biochemical species. It can provide insight into such intimate processes as solvent-solute interactions, the structure and dynamics of nucleic acids, and the permeability of membranes.

Many of these measurements are made possible by the fluorescence lifetime, the average time that a molecule spends in the excited state before emitting a photon and returning to the ground state. It is an important and unique feature of an excited state.

Fluorescence lifetimes are very short. Most fluorescence lifetimes fall within the range of hundreds of picoseconds to hundreds of nanoseconds. The fluorescence lifetime can function as a molecular stopwatch to observe a variety of interesting molecular events. An antibody may rotate slightly within its molecular environment. A protein can change orientation. A critical binding reaction may occur. Because the time-scale of these events is similar to the fluorescence lifetime, the measurement of the fluorescence lifetime allows the researcher to peer into the molecule and observe these phenomena.

Autofluorescence (AF) in biochemistry and medicine are used in studying,

* Protein structure and folding
* Protein-antibody interactions
* Donor-acceptor distances
* Enzyme conformation in proteins and membranes
* Dynamics and structure of membranes
* Permeability and ion transport in membranes
* Lipid dynamics in membranes
* Dynamics and structure of nucleic acids
* Photochemistry of vision
* Mechanism of photosynthesis
* Photodynamic therapy

STUDIES ON AUTOFLUORESCENCE (AF) TECHNIQUES

1. **Liang** et al (2000)[44] reported that most of all emissions are due to excitation of tryptophan residues with a few emissions due to tyrosine and phenyl alanine. Due to the higher fluorescence quantum yield of tryptophan, resonance energy transfer from proximal phenyl alanine to tyrosine and from tyrosine to tryptophan, the emission spectrum of tissues containing the three residues usually resembles that of tryptophan. Further, the photochemical characteristics of tryptophan are very much dependent on its microenvironmental conditions. In particular the emission of tryptophan depends upon its solvent polarity. The fluorescence spectrum shifts to shorter wavelength as the polarity of the solvents surrounding the tryptophan residues decreases.
2. **Ueda Y** and **Kobayashi M.** et al (2004)[72] observed that as the lactic-acid concentration becomes dense, the AF peak intensity from elastin and desmosine solutions become wholly weak. They found a similar

reduction in the autofluorescence intensity for nicotinamide adenine dinucleotide (NADH) solutions. Their analysis indicated that the lactic acid causes the conformational change in elastin and the oxidation of NADH, which can be related to changes in the AF properties.

AUTOFLUORESCENCE IN CERVICAL TISSUES

1. **Drezek R** et al (2001)[25] studied the colposcopic sections that were taken using florescent spectroscopy. Autofluorescence images at 380 and 460nm excitation were taken in their study. They found a significant increase in epithelial fluorescence intensity at 380nm excitation in dysplastic tissues compared to normal sections. They suggested that the increase in fluorescence intensity was probably due to reduced nicotinamide adenine dinucleotide. They also found decreased fluorescence intensity at 380nm and 460nm excitation in dysplastic issues, which corresponds to the wavelength of collagen.

AUTOFLUORESCENCE IN OVARY

1. **Heintzelman DL** et al, (2000)[36] studied the autofluoescence of polymorphonuclear leukocytes, monomorphonuclear leukocytes and cervical epithelial cancer cells. They were successful in discriminating inflammation from dysplasia based on high levels of tryptophan in dysplastic cervical epithelial cells.
2. **Brewer M** et al (2001)[12] used fluorescence spectroscopy to study the normal variations within the ovary, benign neoplasms and ovarian cancer. They took autofluorscence readings in wavelengths from 330 to 500nm from patients undergoing oopherectomy. They obtained

promising results from their study.

AUTOFLUORESCENCE IN GIT

1. **Cothren RM** et al (1996)[21] performed an invivo study to obtain autofluorescence spectra during colonoscopy. Their results indicated that autofluorescence spectra could be used to differentiate hyperplastic polyps from normal colonic mucosa.
2. **Zhiwei Huang** et al (2004)[37] used a microspectrophotometer (MSP) system to identify the microscopic origins of tissue autofluorescence in the colon under the excitation of a helium-cadmium laser at 442 nm. Colonic tissue samples (normal: n=8, adenocarcinoma: n=10) were obtained from 12 patients with known or suspected malignancies of the colon. Autofluorescence microscopy revealed that differences in the clinically measured autofluorescence spectra between normal and tumor tissue were mainly due to thickening of the tumor mucosa resulting in a reduced submucosa fluorescence contribution, as well as the increased hemoglobin absorption in tumor tissue.

AUTOFLUORESCENCE IN BREAST TISSUE

1. **Palmer** et al (2003)[52] examined the fluorescence of tryptophan, reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and flavin adenine dinucleotide (FAD) in normal and malignant human breast cells. All of the malignant cells showed a statistically significant decrease in the tryptophan fluorescence per cell relative to that of the normal cells. They concluded that the differences in normal and malignant human breast tissue fluorescence spectra may be attributed in part to differences in the intrinsic cellular fluorescence of normal and malignant breast epithelial cells.

2. **Tara M. Breslin** et al (2004)[11] took optical measurements from 56 samples of malignant and benign breast tissue. Autofluorescence spectra were measured at excitation wavelengths ranging from 300 to 460 nm, and diffuse reflectance were measured between 300 and 600 nm. They found a statistically significant difference in the diffuse reflectance and fluorescence emission spectra of benign and malignant breast tissue.

AUTOFLUORESCENCE IN ORAL CARCINOMA

1. **kolli VR** et al (1995)[40] carried out an in vivo study in 31 patients with oral neoplasms using xenon lamp spectrofluorometer. They observed significant differences between normal and neoplastic mucosa with autofluorescence after statistical analysis.
2. **Chen CT** et al (1996)[17] performed an in vitro study on oral tissues at excitation wave lengths between 270 to 400nm at 10nm interval. They found that at 300nm excitation, the most intense fluorescent peak occurred at 300nm and 470nm emissions. The diagnostic histogram was developed based on the spectral readings which showed that neoplastic oral tissues can be distinguished from normal oral mucosa at 300nm excitation wavelength.
3. **Dhingra JK** et al (1996)[24] used 370 and 410nm excitation wavelengths for diagnosing 13 oral and oropharyngeal patients. 10 healthy volunteers were also included in the study. They observed a prominent fluorescence at 310nm. They were able to diagnose 17 of the 19 lesions with 2 false positive results.
4. **Ganesan S** et al (1998)[28] studied normal and malignant human oral epithelial cells under ultraviolet excitation. They observed a significant difference in the excitation spectra at 340nm emission between normal and malignant epithelial cells.

5. **Vengadasan** et al (1998)[74] studied fluorescent spectroscopy in DMBA induced hamster cheek pouch carcinogenesis model at 405nm excitation. They analyzed emission spectra between 430 to 700nm to characterize the native fluorescence of endogenous fluorophores in various tissues such as hyperplasia, papilloma and early invasive carcinoma. Their results showed spectral differences between these tissues.
6. **Chen CT** et al (1998)[16] performed autofluorescence on normal and malignant human oral tissues at 330nm excitation wavelength. Significant differences in fluorescence intensity were observed at 380 and 460nm emission. He concluded that at 330nm excitation, fluorescence spectroscopy is useful to detect oral malignant lesions.
7. **Gillenwater A** et al (1998)[29] evaluated the clinical potential of fluorescence spectroscopy (a noninvasive technique for assessing the chemical and morphologic composition of tissue) for in vivo detection of oral cavity neoplasia. They observed consistent differences between the fluorescence spectra of abnormal and normal oral mucosa and concluded that fluorescence spectroscopy has the potential to improve the noninvasive diagnosis of oral cavity neoplasia.
8. **Lezlee Coghlan** et al (2001)[20] used the hamster cheek pouch carcinogenesis model to explore fluorescence excitation wavelengths useful for the detection of neoplasia. Their results showed increased fluorescence near 350-370nm and 410nm excitation and decreased fluorescence near 450-470nm excitation with neoplasia. The optimal diagnostic excitation wavelengths identified using this model are 350-370nm excitation and 400-450nm excitation, which are similar to those, identified for detection of human oral cavity neoplasia.

9. **Wei Zheng** et al (2002)[77] used a 5-ALA mediated digitized fluorescence endoscopic imaging system for the early detection of neoplasms in the oral cavity. PPIX fluorescence endoscopy and fluorescence image quantification were performed on 16 patients with known or suspected premalignant or malignant lesions in the oral cavity. Their initial results indicate that the digitized endoscopic imaging system combined with the fluorescence image quantification method and the ratio diagnostic algorithm developed in this study has the potential to significantly improve the non-invasive diagnosis of early oral neoplasms.
10. **Madhuri S** et al (2003)[45] Native fluorescence characteristics of blood plasma were studied in the visible spectral region, at two different excitation wavelengths, 405 and 420 nm, to discriminate patients with different stages of oral malignancy from healthy subjects. The diagnostic potentiality of the present technique was also estimated in the discrimination of malignant subjects from normal and nonmalignant diseased subjects such as liver diseases. In the discriminant analysis performed across the three groups, normal, oral malignancy (including early and advanced stages) and liver diseases, 99% of the original grouped cases and 95.9% of the cross-validated grouped cases were correctly classified.
11. **Diana C.G. de Veld** et al **(**2003)[22] recorded autofluorescence spectra of oral mucosa from 97 volunteers. They observed differences in fluorescence intensity between different locations. These were significant but small compared to standard deviations (SD). Normalized spectra looked similar for all locations, except for the dorsal side of the tongue (DST) and the vermilion border (VB).

Porphyrin-like fluorescence was observed frequently, especially at DST. The remaining locations showed large overlaps.

12. **Majumder. S. K** et al (2003)[47] carried out a study using a N2 laser-based system involving, 25 patients with histopathologically confirmed squamous cell carcinoma of oral cavity. A general multivariate statistical algorithm was developed to analyze and extract clinically useful information from the oral tissue spectra acquired in vivo. The algorithm could differentiate the squamous cell carcinoma of the oral cavity from normal squamous tissue with a sensitivity and specificity of 86% and 63%, respectively towards cancer.

AUTOFLUORESCENCE IN NORMAL TEETH AND DENTAL CARIES

1. **Konig K** et al (1998)[41] studied autofluorescence characteristics of dental caries. They observed that a wide range of carious lesions revealed characteristic emission of endogenous fluorophores with strong fluorescence bands in the red spectral region when excited with 407 nm. Healthy hard dental tissue exhibited no emission bands in the red. The fluorescence spectra, fluorescence excitation spectra as well as the reflectance spectra of carious lesions were found to be typical for fluorescent porphyrins, mainly protoporphyrin IX. A possible source of these porphyrins within carious tissues is bacterial biosynthesis.
2. **Taubinsky IM** et al (2000)[68] studied the autofluorescence spectra from the hard tissues of a tooth, both in normal and pathology. Their results showed that intact and affected hard tissues were greatly different in the integral fluorescent intensity. Dental calculus was

found to produce the most pronounced fluorescent intensity, whereas the carious regions produced a slightly weaker fluorescent intensity. On the contrary, the intact hard tissues of a tooth exhibited the poorest fluorescent intensity.

3. **Banerjee A** et al (2000)[5] an in-vitro study examined the correlation between the distribution of the autofluorescent signal emitted from carious dentine (detected using confocal laser scanning microscopy) and its microhardness, within the depths of human dentine lesions. They concluded that a correlation existed between the zone of autofluorescence and carious dentine that was markedly softened by the carious process. These findings highlighted a possibility that the autofluorescence might be used as an in-vitro, objective histological marker for the softened, carious dentine requiring clinical excavation.
4. **Gallangher R. R** et al (2003)[27] used a 351-nm laser excitation source to perform autofluorescence microscopy of dentin, enamel, and the dentin–enamel junction (DEJ) to obtain information regarding their morphology and spectral characteristics. The emission spectra of these calcified dental tissues were different from one another, and this enabled the DEJ to be imaged and dimensionalized. The DEJ displayed sharp and clearly delineated borders at both its enamel and dentin margins.
5. **Shigetani Y** et al (2003)[66] evaluated the usefulness of autofluorescence for caries detection. Observations from the autofluorescence and EPMA images in the carious lesions correlated between caries diagnosis and demineralized areas with autofluorescence.

6. **Kidd EA** et al (2003)[43] investigated whether a visual scoring system developed for occlusal caries could be applied to proximal lesions. They used stereomicroscope and confocal laser scanning microscope to determine the depth of caries along with autofluorescence technique. Their results showed reasonable correlation between the visual scores and the stereomicroscope histological evaluations for occlusal surfaces and non-cavitated proximal surfaces.
7. **Borisova E.G** et al (2004)[9] investigated the intrinsic fluorescence of carious human teeth, of different stages of teeth demineralization. They found that differentiation between initial tooth demineralization and early stages of caries could be made by the laser-induced fluorescence spectroscopy method.

AUTOFLUORESCENCE OF DENTAL CALCULUS

1. **Kurihara E** et al (2004)[42] investigated the possibility of sub gingival calculus detection using autofluorescence. The autofluorescent images photographed at an excitation of 633 nm provided clear calculus identification in periodontopathic model teeth when a 700 nm band-pass filter or a 700 nm high-pass filter was used. However, fluorescence intensity was masked when bacterial cells or blood clots covered the calculus surface. They concluded that for clinical use, it would be important to remove sub gingival plaque and debris from root surfaces before attempting to detect subgingival calculus and root caries with this manner.

AUTOFLUORESCENCE OF ORAL SUBMUCOUS FIBROSIS

1. **Hsin-ming chen** et al (2003)[18] measured the in vivo autofluorescence spectra of 59 oral submucous fibrosis mucosal sites and compared the measured spectra with autofluorescence spectra obtained from 15 normal oral mucosal samples from 15 healthy volunteers, 5 samples of frictional keratosis on OSF (FHOSF) buccal mucosa and 29 samples of oral leukoplakia on OSF (OLOSF) buccal mucosa. They found that the spectrum of the OSF mucosa had a significantly higher 380nm emission peak and a significantly lower 460nm emission peak than the spectra of NOM, FHOSF and OLOSF samples. They concluded that OSF has a very unique pattern of autofluorescence spectrum which can be used for real-time diagnosis of OSF.
2. **Tsai T et al** (2003)[71] performed autofluorescence spectroscopy for the diagnosis of oral neoplasia in a high-risk population. They characterized the in vivo autofluorescence spectra from oral submucous fibrosis (OSF) lesions and oral premalignant and malignant lesions in both oral OSF and non-OSF patients. The mean ratio values increased gradually from OSF to normal oral mucosa (NOM), to epithelial hyperplasia (EH) and epithelial dysplasia (ED), and to SCC. Their ANOVA test showed significant differences in the ratio value among all categories of samples ($P<0.01$). They found that EH, ED, and SCC lesions on OSF patients had distorted autofluorescence intensity because of collagen. While the mean ratio values of EH, ED, and SCC between non-OSF and OSF patients showed significant differences.
3. **Wang CY** et al (2003)[76] used a fiber optics-based fluorospectrometer to measure the autofluorescence spectra from healthy volunteers (NOM) and patients with oral lesions of submucous fibrosis (OSF),

epithelial hyperkeratosis (EH), epithelial dysplasia (ED), and squamous cell carcinoma (SCC). They concluded that the PLS-ANN classification algorithm based on autofluorescence spectroscopy at 330-nm excitation is useful for in vivo diagnosis of OSF as well as oral premalignant and malignant lesions.

Summary & Conclusion

In the present study the following salient features were found,

1. Although there are many fluorophores in the tissues, it is found that the emission characteristics of tryptophan, collagen and NADH provides measurable variations when normal tissue transformed into various histological conditions (betel quid chewers mucosa and oral submucous fibrosis).
2. Furthermore, in addition to these fluorophores, the degree of vascularization in oral tissues may also be used as an end point to monitor the tissue transformation.
3. The absorption bands of oxy-haemoglobin at 420nm and 580nm are absent in case of oral submucous fibrosis, which is highly correlating with histopathology reports.
4. Our results showed distinct difference between normal and oral submucous mucous fibrosis.
5. Significant difference in the emission characteristics was also found between betel quid chewers and oral submucous fibrosis patients.

6. However, we were unable to discriminate betel quid chewers from normal individuals, as there is no considerable variation in the spectral signature between them.
7. Although we attributed the decreased fluorescence intensity in oral submucous fibrosis is probably due to the distortion caused by dense fibrosis as advocated by Tsai et al or due to the conformational changes in the collagen molecules of oral submucous fibrosis, further studies are necessary to find the exact reason for the decreased fluorescence.
8. The intensity of oral submucous fibrosis at 390nm is less when compared to normal and betel quid chewers, correlating with the findings of Tsai et al.
9. Many used ratio parameter analysis on complicated statistical methods to discriminate diseased tissue from normal. In the present study we optimized the fluorescence emission intensities at 380 (collagen emission) and 460 (NADH emission) and haemoglobin absorption at 420nm may be used as markers at an excitation of 320nm. The same intensity values were used for statistical analysis in the present study.
10. In the present study we were able to discriminate normal from oral submucous fibrosis with a sensitivity of 100% and specificity of 100%.
11. We were also able to discriminate betel quid chewers from oral submucous fibrosis with a sensitivity of 100% and specificity of 100%.
12. However the discrimination between normal and betel quid chewers was marginal. We assume that with more number of cases and other

sophisticated statistical techniques we can discriminate normal and betel quid chewers.

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