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Ex-vivo PDD to detect malignant cells in oral brush biopsies

Dissertation

zur Erlangung des Grades eines Doktors der Zahnmedizin

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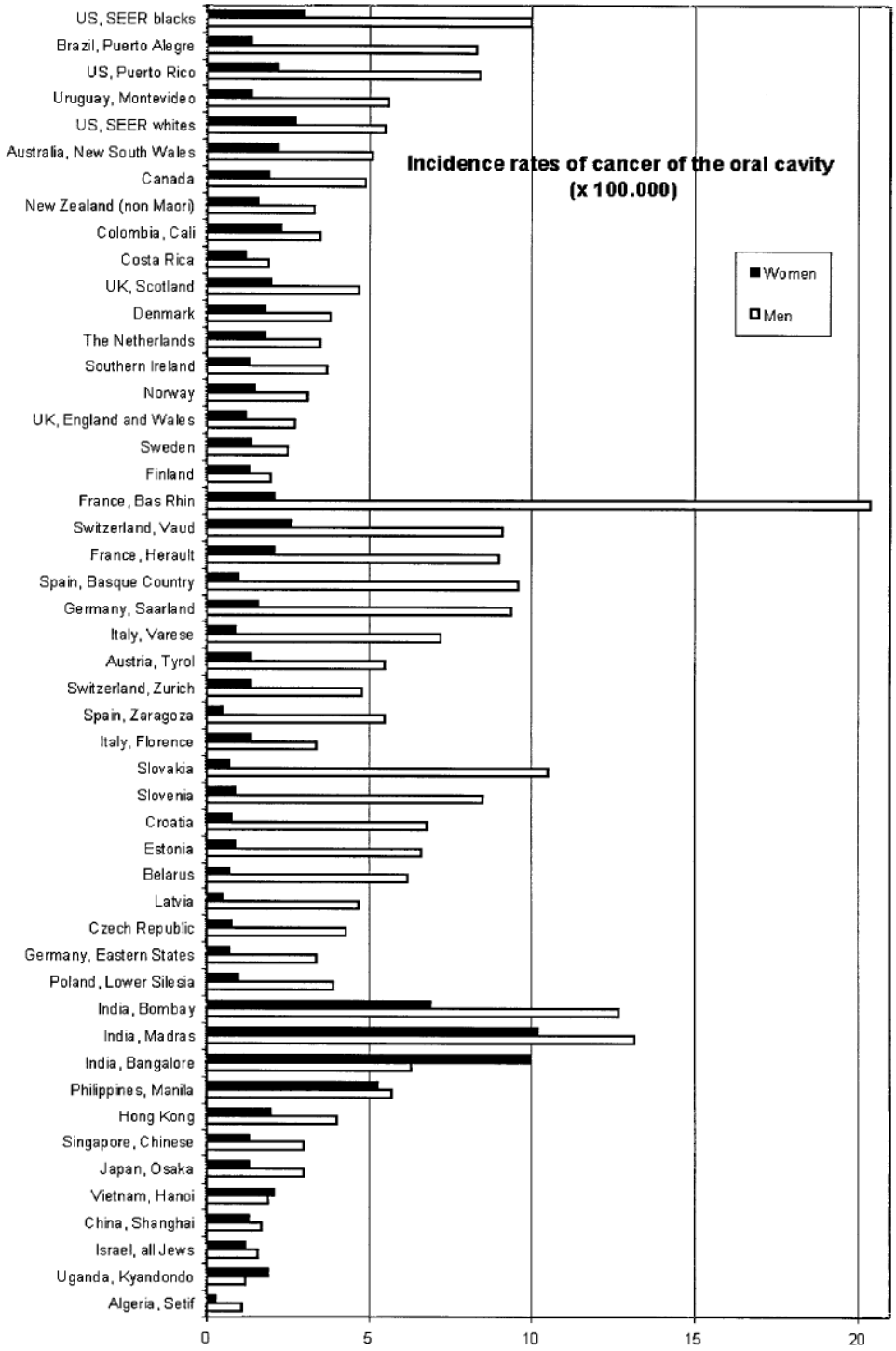
1 Introduction

1.1 Oral Cancer

1.1.1 Epidemiological Facts

Oral squamous cell carcinoma (OSCC) is the most common cancer of the head and neck. Each year it accounts for more than 300.000 cases worldwide, more than 30.000 cases in the United States and more than 3.000 cases in Canada. The 5-year survival rate for OSCC has remained at approximately 50% for the past several decades [1]. Epidemiologic studies have shown significant differences of incidence and prevalence within Europe and the world [2]. The incidence of oral cancer is highest among men in Northern France (49.4/100,000 men), Southern India (more than 20/100,000 men), and some areas of Eastern Europe and Latin America (table 1.1). Under normal circumstances, the oral cavity and oral and oropharynx are comprised of several types of tissues and cells, and tumors can develop from any of theses cells. These tumors may either be benign (they do not spread to the adjoining tissues), or the tumor may invade other tissues of the body. 96% of oral cancers, squamous cell carcinoma is usually preceded by dysplasia presenting as white epithelial lesion on the oral mucosa (leukoplakia). Malignant transformation occurs unpredictably in 1-40% of patients over 5 years [3]. The most common site for intraoral carcinoma is the tongue, which accounts for around 40 percent of all cases in the oral cavity proper. These tumors most frequently occur on the posterior lateral border and ventral surfaces of the tongue. Oral cancer most commonly occurs in middle-aged and older individuals, although a disturbing number of these malignancies is also being documented in younger adults in recent years. Oral cancer most commonly occurs in middle-aged and older individuals, although a disturbing number of these malignancies is also being documented in younger adults in recent years. Tumors are more common among men than women, with a male: female ratio of over 2:1. However, the disparity in the male: female ratio has become less pronounced over the past half century, probably because women have been more equally exposing themselves to known oral carcinogens such as tobacco and alcohol. The floor of the mouth is the second most common intraoral location fig 1.1. Less-common sites include the gingiva, buccal mucosa, labial mucosa, and hard palate[4]. From an epidemiological and clinicopathological perspective, "oral cancer" can be divided into 3 categories: carcinomas of the oral cavity proper, carcinomas of the lip vermilion, and carcinomas arising in the oropharynx. Survival of patients with oral and oro-pharyngeal cancer is strongly related to the stage of disease at diag-

Table 1.1 Incidence rates of oral cancer in the world [2]



-nosis. According to the 1973-to-1988 SEER data from the National Cancer Institute, the five-year relative survival rate for patients with localized disease is 81.9 percent. However, the survival rate drops to 46.4 percent for patients with regional spread and to 21.1 percent for those with distant metastases [5].

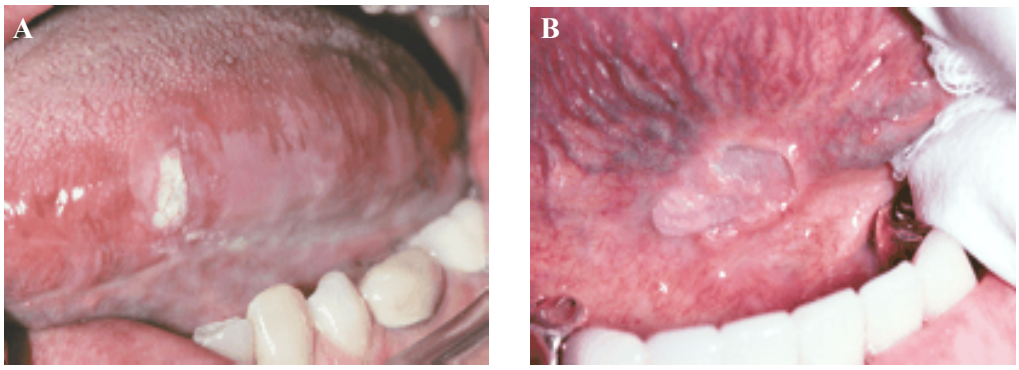


Fig 1.1 (A) A diffuse leukoplakia of the left lateral border of the tongue. A biopsy of the lesion showed early invasive squamous cell carcinoma. (B) Squamous cell carcinoma. Ulcerated lesion of the ventral tongue/floor of mouth [4].

Staging of oral cancer is important for establishing proper treatment and determining prognosis. Tumors are staged using the TNM system, where T represents the size of the primary tumor, N indicates the status of the regional lymph nodes, and M indicates the presence or absence of distant metastases. This system is outlined in Table 1.2 [6]. Metastases from oral squamous cell carcinomas most frequently develop in the ipsilateral cervical lymph nodes. Tumors from the lower lip and floor of mouth may initially involve the submental nodes. Contralateral or bilateral cervical metastases also can occur, especially in tumors of the base of tongue, in advanced tumors, and in tumors that occur near the midline. Involved nodes usually are enlarged, firm, and nontender to palpation. If the tumor has perforated the capsule of the involved node and invaded into the surrounding connective tissue (extracapsular spread), the node will feel fixed and immovable [7]. As many as 30 % of oral cancers have cervical metastases, either palpable or occult, at the time of initial evaluation. In particular, the tongue has a rich blood supply and lymphatic drainage, which accounts for the fact that up to 66 % of patients with primary tongue lesions have neck disease at the time of diagnosis. Distant metastases are most common in the lungs, but any part of the body may be affected [8].

Table 1.2 The TNM classification:

T primary tumor	**N cervical nodes**	**M distant metastases**
Tis carcinoma in situ	N0 no nodes	M0 absent
T1 < 2 cm diameter	N1 single node < 3 cm	M1 present
T2 2-4 cm diameter	N2 multiple ipsilateral nodes or single node 3-6 cm	
T3 > 4 cm diameter	N3 bilateral cervical nodes or ipsilateral node > 6 cm	
T4 massive, invading beyond mouth		

The individual clinical parameters in the TNM classification system are grouped to determine the appropriate disease stage [9]; stages are ranked numerically from 0 (which has the best prognosis) to IV (the worst prognosis) Table 1.3.

Table 1.3 TNM clinical stage grouping:

Stage	Tumor Size	Nodal Involvment	Distant Metastasis
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N1	M0
Stage IV	T1-T3	N0- N3	M0- M1

1.1.2 Etiology of Oral Cancer

The strong association between cancers of the oral cavity and pharynx with tobacco use is well established. Epidemiological studies show that the risk of developing oral cancer is five to nine times greater for smokers than for non-smokers, and this risk may increase to as much as 17 times greater for extremely heavy smokers [10]. The percentage of oral cancer patients who smoke (approximately 80 percent) is two to three times greater than that of the general population. In addition, treated oral cancer patients who continue to smoke have a two to six times greater risk of developing a second malignancy of the upper aerodigestive tract than those who stop smoking. Smokeless tobacco is used in the West, especially in Scandinavia (‘snuff’) and in the USA. Although there are many different preparations of smokeless tobacco (moist or dry snuff, chewing tobacco), there is evidence that some forms of smokeless tobacco have a rather low correlation with oral precancer and cancer. In South and South-East Asia smokeless tobaccos encompasses betel quid. In Northern Africa chewing habits are also prevalent. In contrast to smokeless tobacco used in Scandinavia and the U.S. chewing habits in South and South-East Asia are strongly related with oral cancer, precancer and submucous fibrosis [10]. The fact that oral cancer is still a major health problem on the Indian subcontinent is largely due to the chewing of the betel [2]. Alcohol use has been identified as a major risk factor for cancers of the upper aerodigestive tract. In studies controlled for smoking, moderate-to-heavy drinkers have been shown to have a three to nine times greater risk of developing oral cancer. One study from France showed that extremely heavy drinkers (greater than 100 grams of alcohol per day) had a 30 times greater risk of developing oral cancer (a typical serving of beer, wine, or liquor contains ten to 15 grams of

alcohol) [9]. Other risk factors like Candida albicans, Human papilloma viruses. Candida infections are more common in smokers [11]; precancerous lesions (oral leukoplakia) with Candida infection may carry an increased risk for transformation. Human papilloma viruses types HPV 16 and 18 – well known for their oncogenic potential in cancer of the uterine cervix – are present in oral squamous cell carcinomas in up to 80%. Furthermore there are Specific genes that have been assessed in oral cancer. And in patients with HIV-infection or AIDS beside the Individuals with poor oral hygiene are at slightly increased risk for oral cancer. Dietary factors, such as a low intake of fruits and vegetables, may also be related to an increased cancer risk [12]. Some diseases associated with development of carcinomas, chronic actinic exposure is associated with the development of carcinomas of the lip vermilion. A number of studies have suggested that oral lichen planus, especially the erosive form, may be associated with an increased cancer risk, it was estimated 1-4% of patients develop carcinomas after 10 years, although other investigators have questioned the strength of this association [13]. Iron deficiency anemia in combination with dysphagia and esophageal webs (known as Plummer-Vinson or Paterson-Kelly syndrome) is associated with an elevated risk for development of carcinoma of the oral cavity, oro-pharynx, and esophagus [13]. Immunosuppression appears to predispose some individuals to an increased risk for oral cancer. Carcinomas of the lip have been reported in a number of kidney transplant patients receiving immunosuppressive medications [14].

1.1.3 Normal oral physiology and oral cancer biology

The mouth is that part of the body bounded by the cheeks, lips, palate, and the floor of the mouth (the area beneath the tongue) fig 1.2 [15]. The nasal cavity is located above the mouth and is separated from the mouth by the hard and soft palate. Within the oral cavity is the tongue, teeth, tissue supporting the teeth (gums and bone), and salivary glands. The pharynx, or throat, is the region located behind the oral cavity. The oral cavity (mouth) is protected by a pair of lips. The lips are highly vascularized, skeletal muscle tissue with an abundance of sensory nerve endings. Lips help retain food as it being chewed and play a role in phonation (the modification of sound). The mouth starts digestion by physically chewing the food by teeth and breaking it down with saliva, the average male mouth holds a volume of about 100ml. The oral cavity is divided in a vestibule, the area "outside" the teeth, and an oral cavity proper [16]. The entire oral cavity is lined by a stratified squamous epithelium. The epithelial lining is divided into two broad types, (1) masticatory epithelium covers the surfaces involved in the processing of food (tongue, gingivae and hard palate). The epithelium is keratinized to

different degrees depending on the extent of physical forces exerted on it. (2) Lining epithelium i.e. non-keratinised stratified squamous epithelium, covers the remaining surfaces of the oral cavity. The structure of the oral mucous membrane resembles the skin in many ways. It is composed of two layers, epithelium and connective tissue component of oral mucosa is termed the lamina propria.

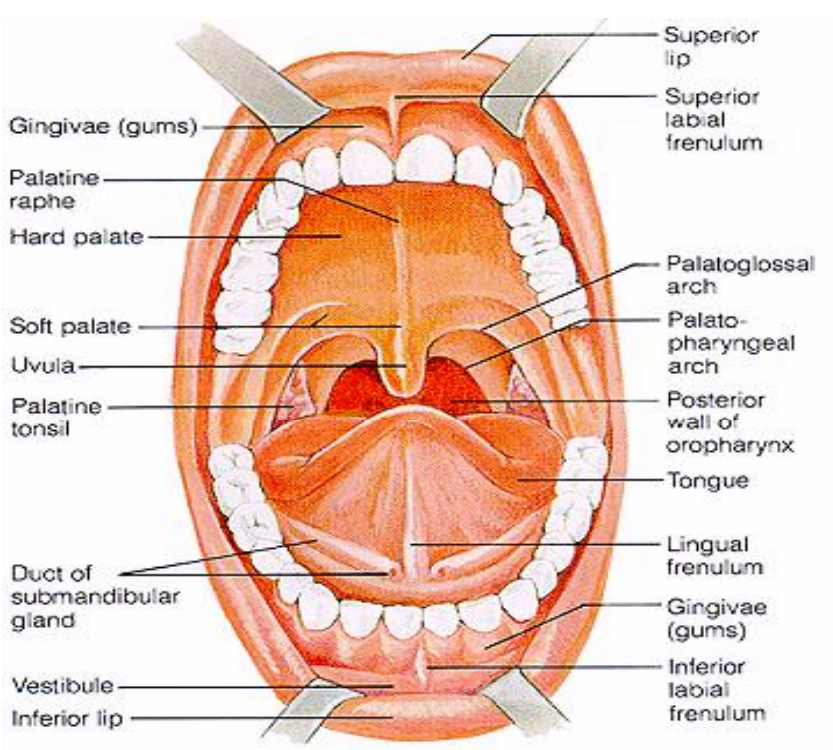


Fig 1.2 Structures of oral the cavity [15]

The comparable part of skin is known as dermis or corium. The two layers from an interface that is folded into corrugation. Papillae of connective tissue protrude toward the epithelium fig 1.3 [17]. The lamina propria may be described as a connective tissue of variable thickness that supports the epithelium. Lamina propria may attach to the periosteum of the alveolar bone, or it may overlay the submucosa, which varies in different regions of the mouth such as the soft palate and floor of the mouth. The submucosa consists of connective tissue of varying thickness and density. It attaches the mucosa membrane to under lying structures. Glands, blood vessels, nerves, and also adipose tissue are present in this layer [18]. The dorsal surface of the tongue is divided by the sulcus terminalis into an oral part, the anterior two-thirds, and a pharyngeal part, the posterior one-third. The dorsal surface of the oral part has a characteristic appearance due to the presence of a large number of small projections, the

lingual papillae fig 1.3. The epithelium of the pharyngeal part forms a somewhat irregular surface which covers the lingual tonsils. The epithelium of the dorsal surface of the tongue rests on a fairly dense layer of connective tissue, which connects the epithelium firmly with the underlying muscular and connective tissues. The muscles of the tongue (skeletal muscle) are organized into strands oriented more or less perpendicular to each other. Their actions provide the tongue with the necessary motility to participate in the formation of speech and to aid in the initial processing of foods. Control of the movement of the tongue muscles and the collection of sensory information necessitate a profuse innervation of the tongue in which a number of the cranial nerves participate (V, trigeminal nerve - sensory - anterior two-thirds; VII, facial nerve-taste; IX, glossopharyngeal nerve-sensory/taste-posterior one-third; XII, hypoglossal nerve-motor).

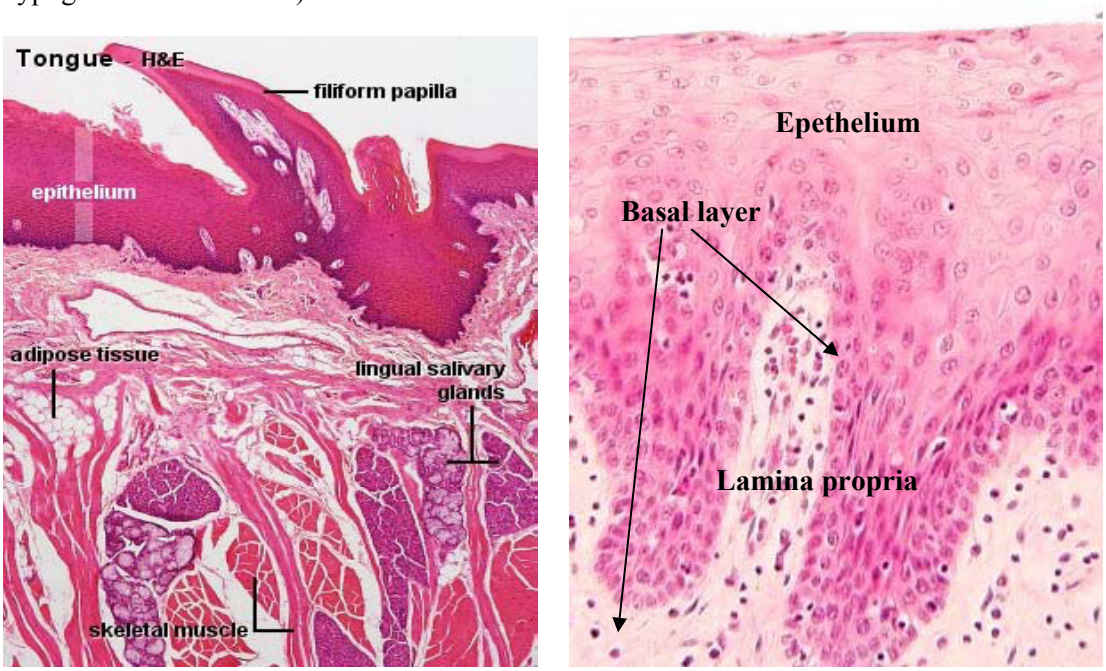


Fig 1.3. Section illustrates the general organisation of the tongue (H&E stain). The small salivary glands located in the connective tissue of the tongue. The papillae on the surface of the tongue, muscular tissue and some of the glands embedded between the muscular tissue of the tongue are visible (left). Oral mucosa of cheek, cells surrounding papillae are basal cells (basal layer), that separate epithelium and connective tissue (submucosa) [17].

Most of the oral cavity carcinogens are chemical (tobacco), physical (radiation) and infectious (papilloma virus, candida) agents which act as mutagens and may cause changes in genes and chromosomes structures by point mutations, deletions, insertions and rearrangements. Many gene alterations have been implicated in the development and progression of oral squamous cell carcinomas and the stages of carcinogenesis have been clearly defined. Expression of genes involved in DNA repair and the stability of the genome is frequently altered. Genetic

changes commonly observed in oral cancers, include loss include loss of heterozygosity (LOH) at the site of known or suspected tumour suppressor genes. The mutations in the tumor suppressor gene P53 are the most frequent genetic altrations in oral cancers, and specific neoplastic marker in OSCC [19]. An increased risk of oral cancer is associated with a number of inherited cancer syndromes, including Li-Fraumeni, Fanconi's anaemia and xeroderma pigmentosum. Some studies have suggested that there is also an inherited component to sporadic oral cancer [19].

1.2 Normal Flora of the Oral Cavity

The presence of nutrients, epithelial debris, and secretions makes the mouth a favorable habitat for a great variety of bacteria. Oral bacteria include streptococci, lactobacilli, staphylococci and corynebacteria, with a great number of anaerobes, especially bacteroides. The mouth presents a succession of different ecological situations with age, and this corresponds with changes in the composition of the normal flora. At birth, the oral cavity is composed solely of the soft tissues of the lips, cheeks, tongue and palate, which are kept moist by the secretions of the salivary glands. At birth the oral cavity is sterile but rapidly becomes colonized from the environment, particularly from the mother in the first feeding. Streptococcus salivarius is dominant and may make up 98% of the total oral flora until the appearance of the teeth (6-9 months in humans). The eruption of the teeth during the first year leads to colonization by streptococcus mutans and streptococcus sanguis. These bacteria require a non-desquamating (nonepithelial) surface in order to colonize. They will persist as long as teeth remain. Other strains of streptococci adhere strongly to the gums and cheeks (epithelial surface) but not to the teeth fig 1.4a [20] and fig 1.4b. In the language of medical microbiologist, a bacterial "adhesin" attaches covalently to a host "receptor" so that the bacterium "docks" itself on the host surface. The adhesins of bacterial cells are chemical components of capsules, cell walls, pili or fimbriae. The host receptors are usually glycoproteins located on the cell membrane or tissue surface. The creation of the gingival crevice area (supporting structures of the teeth) increases the habitat for the variety of anaerobic species found. The complexity of the oral flora continues to increase with time, and bacteroides and spirochetes colonize around puberty [20]. The normal bacterial flora of the oral cavity clearly benefits from their host who provides nutrients and habitat. There may be benefits, as well, to the host. The normal flora occupy available colonization sites which makes it more difficult for other microorganisms (nonindigenous species) to become established. Also, the oral flora contributes to host nutrition through the synthesis of vitamins,

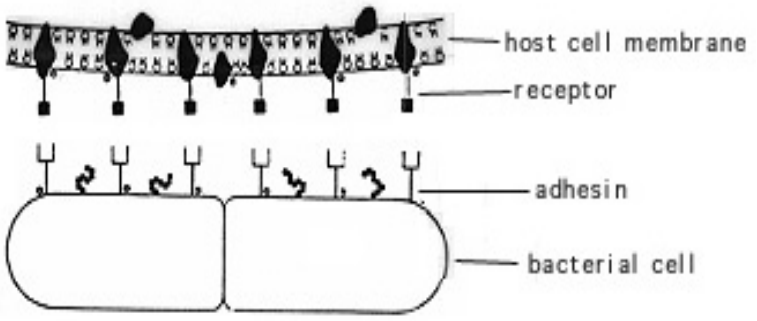


Fig 1.4a Specific adherence involves complementary chemical interactions between the host cell or tissue surface and the bacterial surface [20].

and they contribute to immunity by inducing low levels of circulating and secretory antibodies that may cross react with pathogens. Finally, the oral bacteria exert microbial antagonism against nonindigenous species by production of inhibitory substances such as fatty acids, peroxides and bacteriocins. On the other hand, the oral flora is the usual cause of various oral diseases in humans, including abscesses, dental caries, gingivitis, and periodontal disease. If oral bacteria can gain entrance into deeper tissues, they may cause abscesses of alveolar bone, lung, brain, or the extremities. Such infections usually contain mixtures of bacteria with Bacteroides melaninogenicus often playing a dominant role. If oral streptococci are introduced into wounds created by dental manipulation or treatment, they may adhere to heart valves and initiate subacute bacterial endocarditis [20].

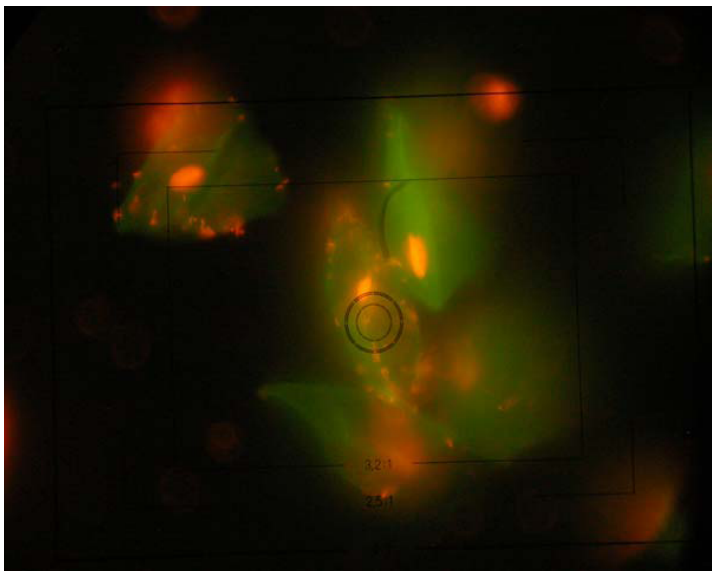


Fig 1.4b Various streptococci adherent to oral epithelial cells in the oral cavity of healthy volunteer. The sample stained by Acridinorange / Ethidiumbromide (green cytoplasm and pale orange nucleus). Colonies of bacteria (commonly are a form of streptococci) are adherent to the surface of epithelial cell (orange color).

1.3 Lung Cancer

Lung cancer is one of the most common diseases worldwide and a leading cause of death in adults. Despite new techniques of detection and treatment, the 5-year survival rate for lung cancer patients continues to be < 15% [21]. The main reason for this low survival rate is that neoplastic lesions are usually detected at a late invasive stage. Every year approximately 46.000 cases of bronchial carcinoma are diagnosed in Germany, and around 40.000 patients die from this disease (Statistisches Bundesamt, 1996) [22]. Lung cancers are of epithelial origin. Epithelial bronchogenic carcinomas can be divided into three categories on a histological basis (Table 1.4): 1. benign, 2. dysplasia and carcinoma in situ, 3. malignant. The malignant tumors (class III) are further subdivided into small cell carcinoma (SCLC) and three other groups: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, which are termed non-small cell lung cancer (NSCLC) according to classification of WHO in 1997.

Table 1.4 World Health Organization histological classification of epithelial bronchogenic carcinoma (1997).

Class I	Benign
Class II	Dysplasia and carcinoma in situ
Class III	Malignant
A.	Squamous cell carcinoma (epidermoid) and spindle (squamous) carcinoma
B.	Small cell carcinoma 1. Oat cell 2. Intermediate cell 3. Combined oat cell
C.	Adenocarcinoma 1. Acinar 2. Papillary 3. Bronchoalveolar 4. Mucus-secreting
D.	Large cell carcinoma 1. Giant cell 2. Clear cell

small cell carcinoma (OAT cells) is considered distinct from the other lung cancers, called non–small-cell lung cancers (NSCLCs). In march 1975, tumor cells isolated from a lung of a patient, whose case was diagnosed as oat cell carcinoma with Eaton-Lambert syndrom (myasthenic syndrom), were transferred to tissue culture [23]. Small cell carcinomas account for approximately 20-25% of all lung cancer. because of their clinical and biologic characteristics. SCLC exhibits aggressive behavior, with rapid growth, early spread to distant

sites [24]. The predominant cause of SCLC, as of NSCLC, is tobacco smoking. Of all histologic types of lung cancer, in fact, SCLC and squamous cell carcinoma have the strongest correlation to tobacco. Small cell carcinomas arise in peribronchial locations and infiltrate the bronchial submucosa. Widespread metastases occur early in the course of the disease. Approximately 65-70% of patients with SCLC have disseminated disease at presentation. Extensive-stage SCLCs are incurable, and patients with extensive disease have a median survival duration of less than 1 year. Even patients presenting with localized disease (ie. limited stage) have a median survival duration of less than 2 years. The 5-year survival rate for SCLC is less than 20%. SCLC typically are centrally located, arising in peribronchial locations. The tumor is composed of sheets of small, round cells with dark nuclei, scant cytoplasm, fine granular nuclear chromatin, and indistinct nucleoli, fig 1.5 [25].

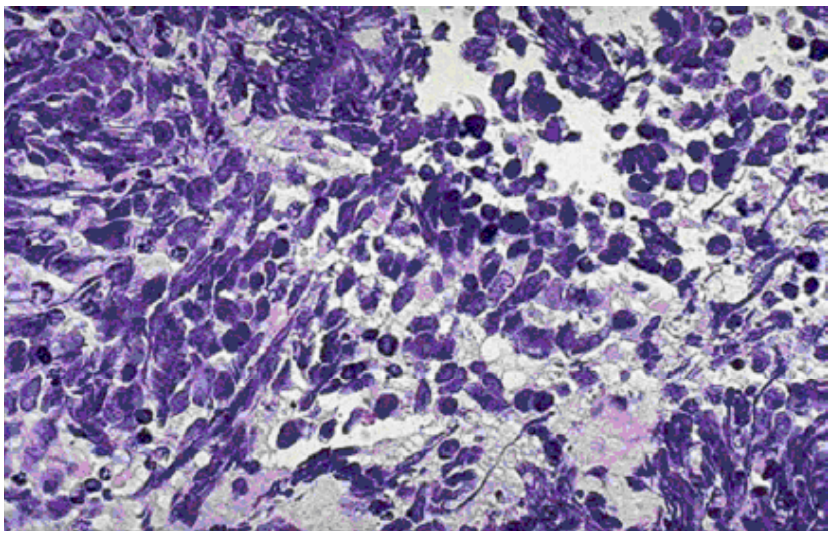


Fig 1.5. Microscopic pattern of a small cell anaplastic (oat cell) carcinoma, in which small dark blue cells with minimal cytoplasm are packed together in sheets [25].

According to (TNM) system 2-stage system used for SCLC initially was proposed by the veterans Administration Lung Group, Limited stage and Extensive stage. Limited stage, the disease confined to one hemithorax; includes involvement of mediastinal, contralateral hilar, and/or supraclavicular and scalene lymph nodes. Malignant pleural effusion is excluded. The disease has spread beyond the definition of limited stage, or malignant pleural effusion is present. The retinoblastoma (RB) tumor suppressor gene, is on chromosome 13 (13q14), and a high percentage of SCLCs (as many as 60%) do not express RB messenger ribonucleic acid (mRNA). This high frequency of inactivation of a tumor suppressor gene suggests that this may be an important step in the molecular pathogenesis of SCLC. The most common

molecular abnormality, however, is deletion of part of chromosome 3 (3p14). Mutations of the p53 tumor suppressor gene are found commonly in both SCLC and NSCLC, but their precise role in pathogenesis is not clear. Tobacco smoking and radon exposure are associated with p53 gene mutations.

1.4 Urinary Bladder Carcinoma

Bladder cancer that forms in tissues of the bladder (the organ that stores urine), Worldwide statistics vary and are inaccurate since renal pelvis tumors are not reported separately. The highest incidence is found in Balkan countries (Bulgaria, Greece, Romania, Yugoslavia), where UCs account for 40% of all renal cancers and are bilateral in 10% of cases. Most bladder cancers are transitional cell carcinomas (cancer that begins in cells that normally make up the inner lining of the bladder). Other types include squamous cell carcinoma (cancer that begins in thin, flat cells) and adenocarcinoma (TCC) that begins in cells that make and release mucus and other fluids). The cells that form squamous cell carcinoma and adenocarcinoma develop in the inner lining of the bladder as a result of chronic irritation and inflammation. Renal urothelial (transitional cell) carcinoma is a malignant tumor arising from the transitional (urothelial) epithelium lining of the renal pelvis [26]. SW1710 (human Caucasian bladder carcinoma transitional cell) epithelial-like, elongated cells fig 1.7 [27], growing adherently as monolayers human urinary bladder transitional cell cacrinoma established from the bladder tumor of an 84-year-old Caucasian woman following transurethral tumor resection in 1977.

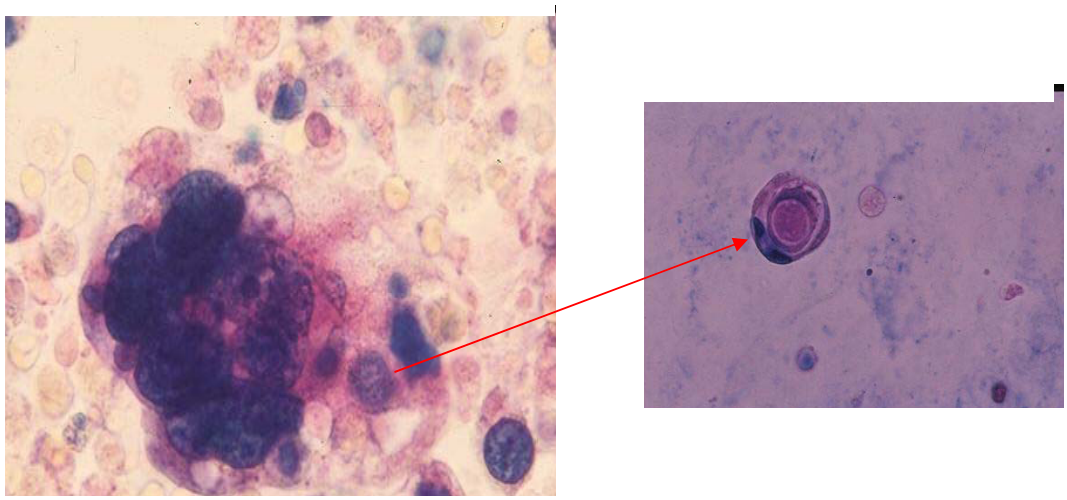


Fig 1.6 The pathological diagnosis for this patient was transitional cell carcinoma. A large clump of cells with a high nucleocytoplasmic ratio and enormous nuclei that are hyperchromatic is seen. Sternheimer stain (right). Atypical transitional cell carcinoma (SW1710 cell) left [26].

Urothelial carcinoma (UC) is the most common tumor of the renal pelvis (UC accounts for more than 90% of renal pelvic tumors) and the sixth most common tumor in man. It arises as a superficial papillary lesion of the epithelium. 75-85% are diagnosed as a superficial stage; 70% of the patient suffer a recurrence after the first treatment and, in 30% the tumor is progressive. The prognosis depends essentially on the prescence of carcinoma in situ and dysplasia [28]. The predominant histological pattern of UC is a papillary tumor with stratified, nonkeratinizing epithelium supported on a thin fibrovascular core. Tumor stage is the most important prognostic factor for upper-tract UC. Survival correlates closely with tumor stage. The TNM staging system of the UICC for upper-tract carcinomas is the most comprehensive (table 1.3).

1.5 Cancer Screening and Early Detection

1.5.1 Cancer screening and diagnosis

Screening is looking for cancer before a person has any symptoms. This can help find cancer at an early stage. When abnormal tissue or cancer is found early, it may be easier to treat. By the time symptoms appear, cancer may have begun to spread. The purpose of screening is to find cancers at an early stage. It is possible, however, to detect some tumors at so early a stage that the biologic propesity to progress and cause death is uncertain. The available technologies for detecting different cancers include different methods and programs according the site of cancer. Imaging modalities, cytologic and molecular evaluation of sputum beside chest x-ray consider as a screening tool of lung cancer. Routine cytoscopy and endoscopic methods are common diagnostic tools of bladder cancer. Recurance and progression of bladder cancer is clearly to the continued prescence of precancerous lesions, of carcinoma in situe, or microfoci in the remaining epithelum. These epithelial lesions are often not visible by cytoscopy and can even be located in epithelium that has a normal appearance. No clinically reliable endoscopic methods are available to localize and determine the complete extent of neoplastic changes [29]. Whereas the inspection is regarded as common examination method of oral cancer. Oral cancer occurs in a region of the body that is generally accessible to visual examination by the patient, the dentist, and the physician. Visual oral examination, including palpation of the tongue, floor of the mouth, salivary glands, and lymph nodes of the neck. Early oral carcinomas are amenable to treatment. Staging generally includes dental x-rays, head-and-chest imaging with radiography, and potentialy ultrasonography, CT, or magnetic resonance imaging (MRI). Disease survival is increased when oral cancers are diagnosed earlier [30]. Leukoplakia and erythroplastic lesions are the

earliest and most serious signs of squamous cell carcinoma. Screening can be made more efficient by inspecting the high-risk sites where 90% of all squamous cell cancers arise, the lower lip is the most frequent site of oral cancer overall, while the tongue is the most frequently affected site within the mouth. In the oral cavity, the majority of cancers are concentrated in the lower part of the mouth, particularly the lateral borders of the tongue, the adjacent floor of the mouth and lingual aspect of the alveolar margin, forming a U-shaped area extending back towards the oropharynx [13]. In addition to thorough physical examinations, physicians attach special attention to the neck and head area. Because of the risk of additional cancers in patients with oral cancers, other parts of the head and neck including nose, larynx, lymph nodes are carefully examined by panendoscopy such as laryngoscopy, pharyngoscopy, or bronchoscopy . Computed tomography that scans parts of body in cross section, is carried out after administration a day that aid in locating abnormalities. This help in judging the extent of cancer spread to lymph nodes, lower mandible and neck. Toluidine blue (vital staining) also is a useful adjunct to clinical examination and biopsy. The mechanism is based on selective binding of the dye to dysplastic or malignant cells in the oral epithelium. It may be that toluidine blue selectively stains for acidic tissue components and thus binds more readily to DNA, which is increased in neoplastic [9]. A definitive diagnosis requires a biopsy of the tissue. Biopsies may be obtained using surgical scalpels or biopsy punches and typically can be performed under local anaesthesia. The incisional and excisional biopsies (scalpel biopsy) are the removal of a piece of small tissue from an area of the tumor or all the tumor respectively, followed by histological evaluation under the microscope. Up to now scalpel biopsy with histological assessment seems to be the only accepted method to definitely evaluate suspicious oral lesions. But most dentists do not have the proficiency to perform biopsies of oral lesions themselves and a few patients may be perilous to suffer a scalpel biopsy. Furthermore literature shows insufficient inter and intra-individual reproducibility of histological grading epithelial dysphasia and unequivocally identifying carcinoma in situ [31-32]. Both CT and MRI have limitations as well as advantages. Soft tissue contrast resolution of CT is relatively poor, which makes it difficult to distinguish between tumor and normal muscle. CT also may require the administration of intravenous contrast material to differentiate vessels from lymph nodes, thereby increasing the risk of an allergic reaction. MRI is has several advantages over CT, MRI can be obtained in multiple planes, which is often helpful in assessing tumor volumes. MRI is less well tolerated patient´s movement than with CT. In addition, although the soft tissue contrast is superb with MRI, fine bone detail is inferior to that obtained with

CT [9]. The analysis of toluidine blue staining in oral cancer screening found that its sensitivity ranged from 93.5% to 97.8%, and specificity from 73.3% to 92.9%. The disadvantages of toluidine blue include the risk of obtaining a false negative reaction in a case where the patient is not followed up adequately. In contrast, the infrequent false-positive only subjects the patient to a biopsy, further more the possibility of allergy. Cell scrapings serves as an adjunct to clinical diagnosis, as it enables more extensive screening and provides microscopic material if there is a delay in or contraindication to biopsy. However, cytologic smears are used infrequently, and patients are not treated on the basis of cytologic findings alone. Smears are most helpful in differentiating inflammatory conditions, especially candidiasis, from dysplastic or neoplastic surface lesions. In addition, cytology may be helpful in detecting field change in oral cancer, especially if this method is used in conjunction with vital staining. Cytology may also be helpful when ulcerations following radiation are suspicious and biopsy is delayed. Fine needle aspiration biopsy of subsurface masses is also an accepted diagnostic test, one that has increased in popularity over the past few years. This technique is extremely useful in evaluating clinically suspicious changes involving salivary glands and lymph nodes.

1.5.2 New techniques for early detection Of cancer

1.5.2.1 Oral brush biopsy with computer-assisted analysis

At the present time, the most effective approach to reduce morbidity and mortality from oral cancer is early detection. In spite of great surgical, chemo-and radiotherapeutical efforts the 5-years survival rate could not be decreased so far, being still less than 50% [33]. However oral cancer can be cured, if detected and treated early enough. Nowadays, an alternative method in order for the examination of suspicious oral mucosal lesion is exfoliative cytology. It is principally based on the method of Papanicolaou (Dr. George Papanicolaou, 1954), which has been acceptd worldwide since 50 years, as a successful method in order to screen for epithelial dysplasia, in situ or invasive carcinomas of the uteri cervix [34]. Additionally, a tool adjuvant to the cytological diagnosis of oral mucosal smears: DNA image cytometry has been recently introduced for the very early diagnosis of malignant transformation of squamous epithelial cell [35-37]. The oral brush biopsy is a new procedure and is defined as collection of oral disaggregated transepithelial cells via rotational brushing of the oral mucosa. The brush turned on glass slides which were stained according to Papanicolaou and examined according to accepted cytological criteria for dysplasia and malignancy [38]. The reliability of the different instruments used in oral exfoliative cytology has been analysed in different

studies [39]. The instrument used for making cytologies should be easy to use in any locations, cause minimum trauma and provide an adequate and representative number of epithelial cells. It has been shown that a brush is an adequate instrument due to its ease in sampling and to the quality of the oral cytologic sample. Brush biopsy is a simple, relatively inexpensive, high sensitive, risk-free method of screening for cancer and serves as an aid to the clinical examination. However, more independent studies analysing its true validity and reliability as well as its applicability and its improvement with other techniques are necessary. It is important to mention that it shall never prevail over the classic biopsy and in all clinically suspicious lesions even with a benign cytologic diagnosis a biopsy should be done [40]. The importance of brush biopsy has been recently emphasized in a multicenter study where nearly 5% of clinically benign-appearing mucosal lesions were sampled by this technique and later confirmed by typical scalpel biopsy to represent dysplastic epithelial changes or invasive cancer [41]. Other authors have also demonstrated the ability of the brush biopsy to uncover similar type lesions that were not clinically suspicious for carcinoma or preinvasive disease [38]. There are controversies related to the real value of this technique in the early detection of oral sequamous cell carcinoma (OSCC). The existence of false positives has been pointed out showing high sensitivity (90%) and low specificity (3%) [42].

1.5.2.2 Photodetection by the Use of 5-aminolevulinic acid

1.5.2.2.1 History of photodetection and phototherapy

Light has been employed in the treatment of disease since antiquity. Many ancient civilizations utilized phototherapy, but it was not until early last century that this form of therapy reappeared. Following the scientific discoveries by early pioneers such as Finsen, Raab and Von Tappeiner, the combination of light and drug administration led to the emergence of photochemotherapy as a therapeutic tool. The isolation of porphyrins and the subsequent discovery of their tumor-localizing properties and phototoxic effects on tumor tissue led to the development of modern photodetection (PDD) and photodynamic therapy (PDT). This review traces the origins and development of PDD and PDT from antiquity to the present day. Porphyrins were identified in the mid-nineteenth century, but it was not until the early twentieth century that they were used in medicine. Hematoporphyrin was first produced by Scherer in 1841 during experiments investigating the nature of blood. Dried blood was heated with concentrated sulfuric acid, the precipitate was washed free of iron and then treated with alcohol. However, the fluorescent properties of hematoporphyrin were not described until 1867 and it was named hematoporphyrin in 1871 [43]. Hausmann in Vienna

performed the first studies of the biological effects of hematoporphyrin. In 1911, he reported on the effect of hematoporphyrin and light on a paramecium and red blood cells and described skin reactions in mice exposed to light after hematoporphyrin administration. In particular, he described acute, subacute and chronic photosensitivity changes and some phototoxicity with intense light. The first report of human photosensitization by porphyrins was in 1913 by the German, Friedrich Meyer-Betz. In order to determine whether the same effects could be induced in humans as well as mice, he injected himself with 200 mg of hematoporphyrin and subsequently noticed prolonged pain and swelling in light-exposed areas.The first report of fluorescent porphyrin localization in a malignant tumor appeared in 1924 when a Frenchman, Policard, from Lyon observed the characteristic red fluorescence of hematoporphyrin in an experimental rat sarcoma illuminated with ultraviolet light from a Woods lamp. Although the fluorescence was correctly attributed to porphyrin localization within the tumor, it was initially thought to be caused by secondary infection, as similar fluorescence had been observed in bacterial cultures. There were no further publications until 1942 when Auler and Banzer from Berlin described the localization and fluorescence of exogenously administered porphyrins in malignant tumors. In 1948 Figge and Weiland administered a range of porphyrins, including hematoporphyrin, coproporphyrin, protoporphyrin and zinc hematoporphyrin, to 240 mice with experimentally-induced and transplanted tumors and 50 non-tumor-bearing mice. The fluorescence was not seen in normal tissues, other than lymph nodes, omentum, fetal and placental tissue and healing wounds. During the war years the first reports of attempts to localize human tumors with fluorescent porphyrins appeared in the early 1950s. In 1951, Manganiello and Figge studied the effects of hematoporphyrin in three patients with head and neck malignancies but fluorescence was not detected. This failure was ascribed to the proportionately lower doses of photosensitizer given to humans, as compared with those in previous animal experiments [43]. In 1955, Rassmussan-Taxdal studied the effects of intravenous infusions of hematoporphyrin hydrochloride administered to patients before the excision of a variety of benign and malignant lesions. Typical red fluorescence was observed in seven out of eight malignant tumors but in only one of the three benign lesions. Tumor fluorescence increased in proportion to hematoporphyrin dose, and with higher doses it was possible to detect a breast cancer through intact skin and a colonic adenocarcinoma through the bowel wall. The authors concluded that this finding had major implications for tumor diagnosis [43]. In 1971, two otolaryngologists, Leonard from Philadelphia and Beck from Iowa, reported a study of tumor detection using hematoporphyrin deriative (HpD) in 40 patients with suspected head and neck tumors. The typical red fluorescence of

hematoporphyrin was observed in 29 patients with biopsy proven malignancy, furthermore, in 5 patients the hematoporphyrin fluorescence was used to aid detection of the lesions and the choice of biopsy site. in 1979, a krypton ion laser was developed, with a 405 nm wavelength to excite porphyrin during endoscopy. The potential application of (HpD) fluorescence using a krypton ion laser for localization of early lung cancer was demonstrated in an animal model by Hayata and Dougherty. In 1982, Hayata also used a similar system to study 36 patients with bronchial neoplasms and four with metaplasia. In 1978, Dougherty reported the first large series of patients successfully treated with PDT. In 1982 The first report by Hayata described a significant bronchoscopic response in the majority of patients, but only one patient of 14 was cured. Following Kelly's early report of PDT of a bladder tumor using hematoporphyrin derivative many clinical studies have assessed the use of PDT in transitional cell carcinomas of the bladder. In 1983 Benson reported four cases of in situ carcinoma responding to PDT, and Ohi and Tsuchiya published a series of 11 superficial tumors successfully treated using light delivery via a flexible cystoscope. In 1987, Prout treated 19 patients with bladder tumors of whom nine (47%) had a complete response with 37 of 50 individual tumors eradicated. More recently, in 1995, Sibille published a large series of 123 patients with esophageal cancer treated with PDT. The previously described studies had demonstrated the potential role of hematoporphyrin as a diagnostic tool for cancers. However, a major disadvantage was the large dose necessary to produce consistent photosensitizer uptake, which also led to unacceptable phototoxicity. In 1955, Schwartz [44] demonstrated that the hematoporphyrin used in previous studies was a mixture of porphyrins, each with different properties. He showed that, after partial purification, the pure hematoporphyrin produced localized only very poorly in tumors, whereas the residue left behind had great affinity for tumor tissue. Schwartz continued his experiments in an attempt to further purify this non-hematoporphyrin fraction. Amongst other processes he treated crude hematoporphyrin with acetic and sulfuric acids, filtering and then neutralizing with sodium acetate, before redissolving the precipitate in saline to produce a substance which became known as hematoporphyrin derivative (HpD). This substance was found to be approximately twice as phototoxic as crude hematoporphyrin, having a lethal effect on mice subsequently exposed to light. The nature of the reaction was similar to that previously demonstrated by Hausmann with skin irritation, edema and erythema, leading to skin necrosis and death. Animals kept in the dark suffered no ill effects. The severity of the reaction was dependent on three factors, the drug dose, the duration of light exposure and the time interval between drug administration and light exposure.

1.5.2.2.2 Photosensitizing agents

The majority of photosensitive molecules have a heterocyclic ring structure similar to that of chlorophyll or hemoglobin. An ideal photosensitizer (PS) must be biologically stable, photochemically efficient, selectively retained in the target tissue relative to surrounding normal tissue and should have low incidence of administrative toxicity (i.e hypotension or allergic reaction). Further more, it should have low levels of dark toxicity to both human and experimental animals. The PS should absorb light in red or far-red wavelengths, in order to pentrate tissue. They should be rapidly eleminated from patient (less than one day). Ideally, water soluble or soluble in harmless aqueous solvent mixture [45]. The majority of photosensitizers are derivatives of hematoporphyrin, a synthetic porphyrin synthesized from heme. In 1983, Dougherty [44] demonstrated that crude hematoporphyrin contains a range of different porphyrins and, when converted to HpD by acetylation further porphyrins are produced, such as protoporphyrin and hydroxyethylvinyldeuteroporphyrin. The following year he proposed that the active component of HpD was composed of two porphyrin units linked by an ether bond. The active component of HpD comprised a mixture of porphyrin rings, between 5 and 8, linked by a number of ether and ester bonds. It is available commercially as "Photofrin(R)" (porfimer sodium, Axcan Pharma, Montreal, Canada), a heterogenous mixture of porphyrins, many of which are not active as tumor sensitizers. Although Photofrin is the most commonly used photosensitizer it has significant side effects. Therefore, major effort has been invested in the development of new sensitizers. In particular, there was a need for new compounds that absorb light at longer wavelengths to assist tissue penetration, greater PDT efficiency, selective tissue localization and self-limiting minor skin photosensitivity. To this end many other sensitizers have been described such as, Phthalocyanines (may be produce a superficial necrosis without underlying muscle damage following light administration). Meso-tetra (hydroxyphenyl) porphyrins (unacceptable degree of skin photosensitization and damage to underlying muscle layers when used in the treatment of epithelial lesions). 5-Aminolevulinic acid (ALA has significant potential advantages over HpD and other photosensitizers, including more rapid photosensitizer clearance, leading to a shorter period of skin photosensitivity, usually no more than 24 h [46] and oral administration. It has been shown to produce selective photosensitizer accumulation in the mucosa of hollow organs and may, therefore, be a superior photosensitizer for the treatment of dysplastic or noninvasive disease). Texaphyrins (as a therapy it was well tolerated and there were few side effects, including transient parasthesias and minor self-

limited skin rashes). Tin ethyl etiopurpurin (there was an incidence of skin photosensitivity of 10-15% at one or more months after treatment). Benzoporphyrin derivative such as Verteporfin and Visudyne (they are cleared relatively quickly from the body, and patients are instructed to avoid direct sunlight and wear sunglasses with a low (4%) transmittance of visible light for 2-5 days after treatment), N-aspartyl chlorin e6 (no significant side effects other than transient skin photosensitivity. This is despite the drug remaining in the plasma for up to 6 weeks) [43].

1.5.2.2.3 5-aminolevulinic acid induced protoporphyrin IX

For 40 years, photodynamic diagnosis (PDD) has investigated the decrease in risk of under-diagnosing tumours. After some drawbacks, the first clinical report of 5-aminolevulinic acid (fig 1.6) as a photosensitising agent in the early 1990s enlivened the interest in PDD [47]. Kennedy were the first to propose 5-ALA induced protoporphyrin IX for the detection of pre-malignant and malignant oral mucosa. The principles of fluorescence diagnosis of tumors are as follows: a. HpD or Photofrin emits a red fluorescence when excited by violet light, and this can be detected by sensitive imaging devices; and b. the concentration of HpD or Photofrin in malignant tumors is higher than in most nonmalignant tissue, thus tumors can be detected by their more intense fluorescence. However, the use of fluorescence detection with synthetic porphyrin mixtures still has certain limitations in diagnostic effectiveness mainly, due to side effects such as skin photosensitization and interference by tissue autofluorescence (Dougherty *et al.*, 1990) [43]. Therefore, new substances like protoporphyrin IX (fig 1.7) are being investigated. Protoporphyrin is an intermediate product in the haem cycle, and is the origin both for the characteristic dual-peaked porphyrin fluorescence at 635 and 700 nm and photosensitisation of living tissue following 5-ALA administration. ALA-PDT was first applied to humans by Kennedy et al. (1990) and has been successfully used for PDT of skin malignancies by Kennedy et al. (1992), and Svanberg et al. (1994). Diagnostic techniques using porphyrin fluorescence as a tumour marker have been developed. These techniques have been developed in parallel with photodynamic therapy utilising porphyrin photosensitisation of various types of malignancies by (Profio 1990, Andersson-Engels and Wilson 1992), [48]. In most clinical and preclinical studies systemic or topical application of 5-ALA is used to temporarily increase the accumulation of porphyrins, i. e., in particular PpIX in the target tissue. The biosynthetic pathway of heme consists of eight discrete enzyme-catalyzed steps, which are distributed between the mitochondrial and the cytosolic compartments of the cell.

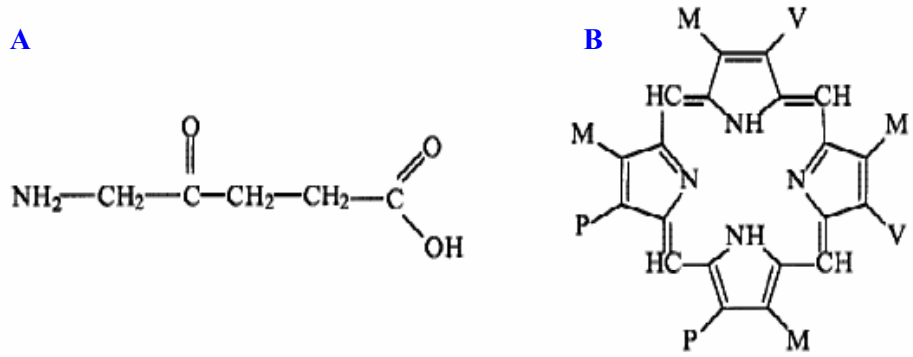


Fig. 1.7 A. Structure of 5-Aminolevulinic acid (5-ALA). B. Structure of Protoporphyrin IX (P=Proprionylethyl-, M=Methyletyhl-, V=Vinylethyl-groups). [46]

The 5-ALA is a precursor of heme formed by 5-ALA- synthase from glycine and succinyl-CoA, the rate limiting step of the heme biosynthesis (fig. 1.8). Once this step is bypassed by exogenous administration of 5-ALA, formation and accumulation of PpIX preferentially takes place in tissues known for high cellular turnover. To date 5-ALA-induced fluorescence has been successfully employed in diagnosis and treatment of various premalignant and malignant diseases. As a given condition for both techniques photoreactive drugs accumulate mainly in tumors and other hyperproliferating tissues such as dysplastic lesions. The main reason why PpIX accumulates with some degree of selectivity is still unknown. But it supposed that peculiarities of blood circulation, enhanced vascular permability, low rate of lymphatic drainage and distinct drug uptake by the tumor cells maybe the factors influencing this process [49]. Others have suggested that the major cause of selective accumulation of PpIX in tumor cells is a reduced activity of ferrochelatase and high activity of the enzyme porphobilinogen deaminase. This enzyme catalyses physiological incorporation of iron into PpIX with formation of heme [50]. The ferrochelatase activity is reduced 2-10 fold in number of different tumors compared with healthy tissue. The low metabolic activity of ferrochelatase in cancer cells is probably due to the prevalence of glycolysis rather than the oxidative phosphorylation found in normal tissue [50]. 5-ALA-induced fluorescence diagnosis of tumors is based on differences in the metabolism of tumors and adjacent healthy tissue. The clinical use of 5-ALA is attractive for two reasons. First, PpIX preferentially accumulates in tumor tissue to a greater extent than other photosensitizing drugs, thus minimizing damage to normal surrounding tissues during treatment. Second, PpIX is rapidly cleared from the body, resulting in skin photosensitization lasting only 48 h. The use of exogenous 5-ALA for tumour detection has been investigated for various organs. For the oral cavity, some promising results have been obtained. However, the use of exogenous 5-ALA has some major

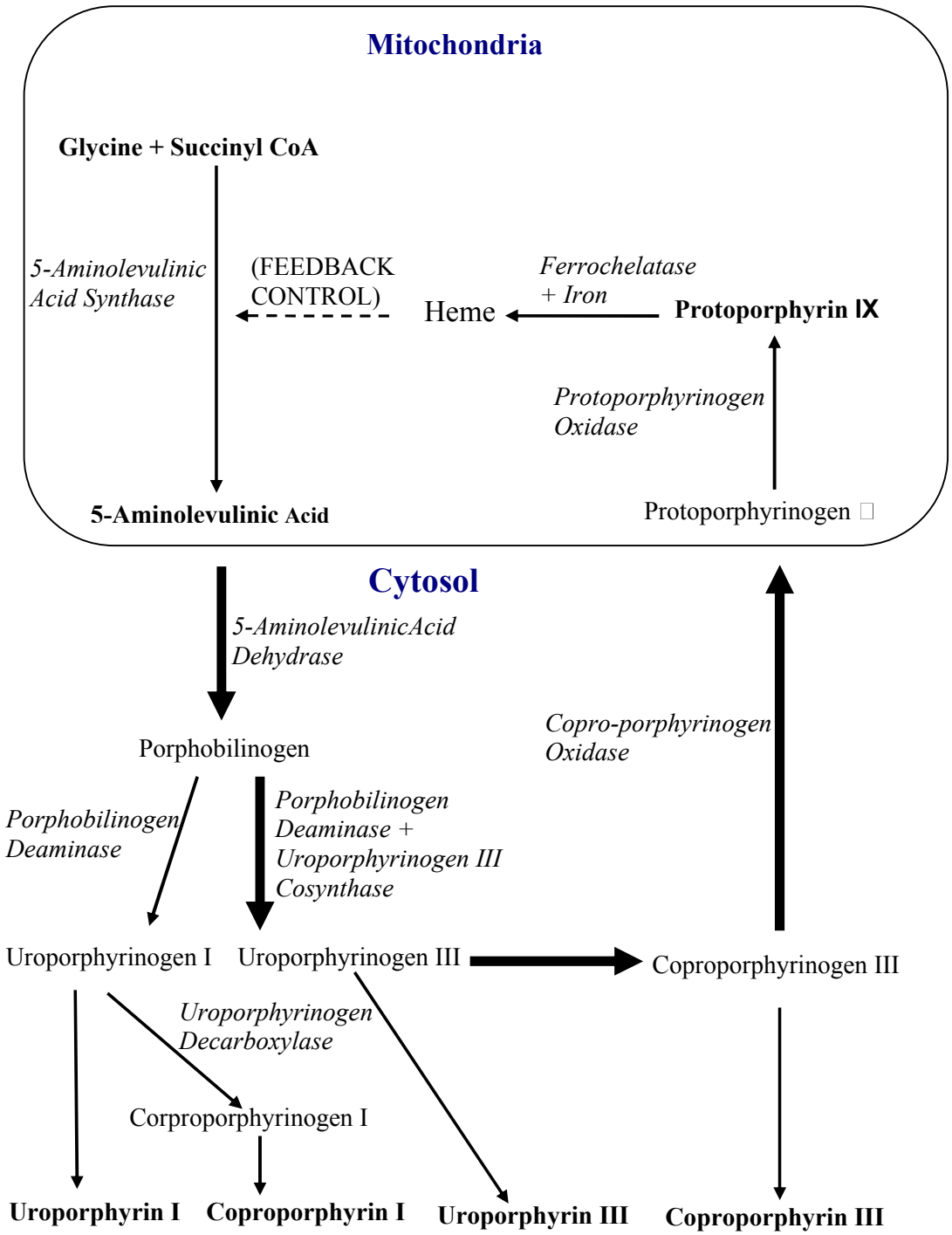


Fig. 1.8 Simplified biosynthetic pathway for heme. Fluorescing and photosensitizing compounds are indicated by big arrow. The 5-aminolevulinic acid/heme feedback control is indicated by a dashed arrow. The principal biosynthetic route for 5-ALA induced PpIX is indicated by the large arrows.

drawbacks. A certain waiting time after application is necessary for the 5-ALA to reach its optimal fluorescence intensity. Furthermore, the application of photosensitizers leaves the patient temporarily sensitive for light, which negatively affects his daily life. This makes the technique impractical, especially for use in regular screenings of high-risk patient groups.

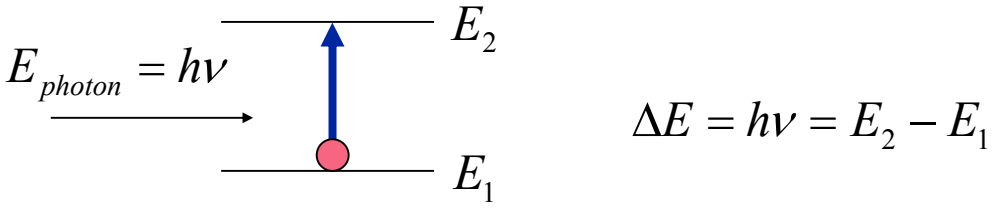
1.5.2.2.3 Influence of light on PpIX and fluorescence kinetics (Photobleaching)

When electrons of the PpIX molecule are excited by blue-violet light (λ=400-410 nm), the energy absorbed is released mainly by emission of red light (fluorescence) (fig 1.9). Since the excited PpIX molecule already loses some of the excitation energy before it fluorescence, the fluorescence light (λ=635 nm) has a longer wavelenght than that of the exitation light (λ=400-410 nm), i.e. it has a red instead of a blue color. There is a lower secondary emission peak in the far red zone around 700 nm. When sufficiently intense, these wavelengths can be visualized by endoscopic devices that have been modified for exciting and detecting of fluorescence. The intensity of PpIX fluorescence emitted by the tissue surface decreases continuously due to photochemical decomposition of PpIX (Photobleaching) [50]. After excitation with a wavelenght of 400-410 nm, the half-life of this photochemical reaction is approx. 15-20 minutes, with the fluorescence decreasing much more strongly during the first few minutes of irradiation. The rate of Photobleaching also depends on the intensity of irradiating light, it has been shown in clinical practice hat only 2-10 minutes are available for intravesical fluorescence diagnosis of each lesion.

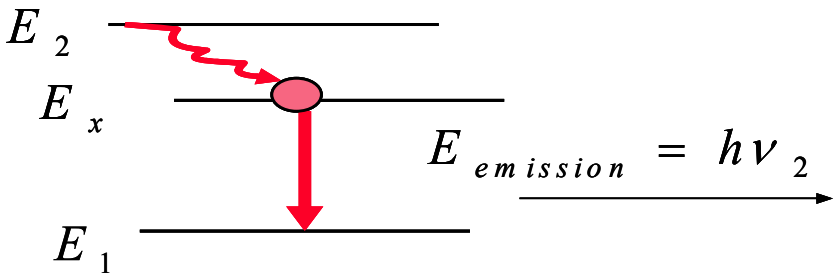
1.6 Autofluorescence

The fluorescence characteristics of tissues depend upon their biochemical composition and histomorphological architecture, both of which undergo a change during malignant transformation. These changes are detectable as an alteration in the fluorescence spectral profile of the tissues [52]. Detection of dysplasia and carcinoma in situ can also be achieved without using any exogenous drug. Policard is considered the first to have recognized the presence of endogenous porphyrins in human tumors in 1924. Later many researchers have extended their investigations of endogenous porphyrins in tumors to the oral cavity [53]. Apart from porphyrins, several naturally occurring tissue components are held responsible for producing the so-called NCF (native cellular fluorescence); each fluorophore's contribution to the overall fluorescence emission depending on the excitation wavelengths. The relevant fluorochromes are mainly localized in the submucosa. In spectrophotometric evaluations, even 94.4% of the tumors showed lower autofluorescence intensities than the sur-

(a) Absorption



(b) Emission



(c)

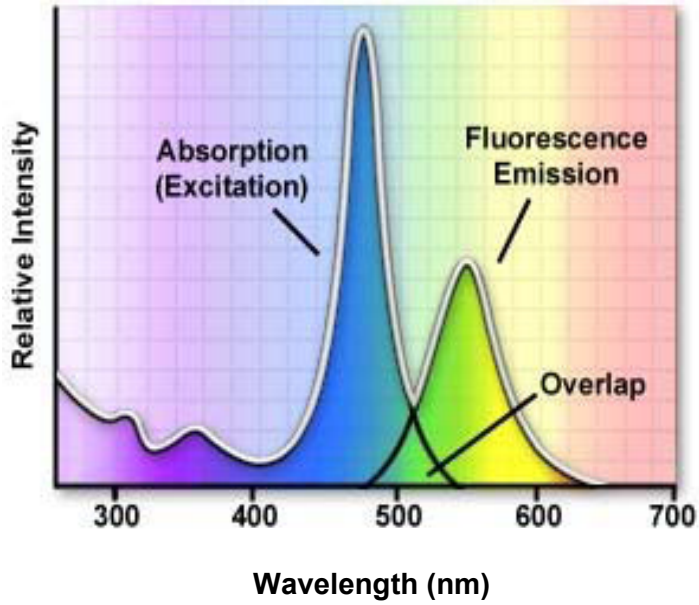


Fig 1.9. The absorption spectrum of a molecule is the absorbance as a function of wavelength (Absorption is fast ≈ 10^{-15} sec) (a). In emission (Opposite of absorption) there is some loss in the molecule, so the emitted energy is lower than the absorbed energy (b). (c) The emission spectrum is characteristic of the molecule, and is independent of the excitation wavelength [51].

-rounding host tissue. The only endogenous fluorophores eligible for being considerably excited at the band of excitation wavelengths have utilized are oxidized flavins (FAD), porphyrins, elastin and partly collagen [53]. When being excited at the used band of wavelengths (375 to 440 nm), all of the fluorophores mentioned above show fluorescence emission in the green spectral range but the porphyrins, which fluoresce in a bright red color.

However, also healthy oral mucosa shows great differences in autofluorescence intensities for different locations and even within the same defined regions of the oral cavity both inter- and intraindividually. The hard palate exhibits much more autofluorescence than other locations in oral cavity [53]. Some studies [Buchalla et al., 2004; Alfano and Yao, 1981] have examined the ability of bacteria to produce red fluorescing compounds. A selection of cultivable bacteria associated with caries and periodontal disease [Marsh and Martin, 1992] were chosen in order to carry out an initial investigation of their potential for fluorophore production [54].

1.7 OBJECTIVES OF THE STUDY

Oral cancer is the fifth most common cancer in the world. Despite advances in surgery, radiation, and chemotherapy, the five-year survival rate for oral cancer has not improved significantly over the past several decades and it remains at about 50 to 55 percent. Despite the improvements in the methods of early screening of oral cancer such as the methods used for collecting oral cytologic material this methodology still presents problems in diagnosing oral cancer. Problems which are mainly due to the existence of false negatives obtained as a result of a non representative sample as well as the subjectivity of the cytologic evaluation. In other hand ALA-based fluorescence diagnosis has restricted specificity due to the prescence of bacteria and anatomical variation of oral cavity. Prompted by the experience of our group and preceding publications on this topic, our in vitro study was performed to achieve:

- To test the efficiency of fluorimetric detection of a minimum number of malignant cells and oral epithelial cells; that were isolated from brush biopsy, (transferred to micro vials).

- To study the kinetics of 5-aminolevulinic acid induced protoporphyrin IX (PpIX) fluorescence in normal oral cell that is scraped from heathy oral epithelium in comparison with different malignant cell lines.

- To determine the most appropriate antiseptic agent to improve the 5-ALA-based fluorescence diagnosis.

In summary, in the present study we investigated whether the combination of PDD with oral brush biopsy, with suppression of bacterial fluorescence, might become a suitable chair side tool to detect early oral squamous cell carcinoma (OSCC).

2 Materials and Methods

2.1 Biological Materials

2.1.1 The tumor cell lines

2.1.1.1 OAT SCLC (Small Cell Lung Carcinoma)

OAT is a highly malignant carcinoma derived from a human bronchial carcinoma. It accounts 15 to 20 % of all lung cancer. OAT 75 has been classified as undifferentiated small-cell lung carcinoma (SCLC). The tumor cell line was isolated from a lung of a 63-year-old male Japanese patient in 1975. Cytological the OAT cells contain a relative large, hyperchromatic nucleus (twice the size of lymphocyte) and scanty cytoplasm [23].

2.1.1.2 SW1710 (Urinary Bladder Carcinoma)

This human urinary bladder transitional cell carcinoma was isolated from the bladder tumor of an 84 year old Caucasian woman in 1977. Several studies have demonstrated that mutations in p53 are common in bladder cancers [55]; [56] Some studies also have suggested a critical role for p53 in tumor progression. The tumour cell line was kindly provided by Prof. W. A. Schulz from Heinrich Heine University, Düsseldorf.

2.1.1.3 HEK293 Cells (Human Embryonic Kidney)

Human Embryonic Kidney cells are an epithelial cell line. Also known as HEK cells, HEK 293 or 293 cells. 293 cells were generated by transformation of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA in the laboratory of Frank Graham in the late 1970s [Graham 1977]. HEK cells are very easy to work with, and so are widely used cell lines in cell biology research.

2.1.1.4 Oral Epithelial Cells and Saliva

The tissue that lines the inside of the mouth is known as the basal mucosa and is composed of squamous epithelial cells. These structures commonly thought of as cheek cells, dorsum of the tongue cells divide approximately every 24 hours and are constantly shed from the body. The epithelial cells can be easily obtained through a mouth rinse, simple brush, or swab. Fifty

samples were collected from healthy oral mucosa by brush. Saliva is the watery mixture of secretions from salivary and oral mucosa glands. Saliva contains electrolytes, mucus, enzymes, and oral flora [57]. Secretion of saliva is under control of the autonomic nervous system, which controls both the volume and type of saliva secreted [58]. The bacteria are the most numerous and obvious microbial components of the oral normal flora. More than 200 species of bacteria include streptococci, lactobacilli, staphylococci, and corynebacteria, with a great number of anaerobes, especially bacteroides. Bacteria that commonly found on the surface of the oral structure are shown in table 1[20].

BACTERIUM	Mouth
Staphylococcus epidermidis	++
Staphylococcus aureus	+
Streptococcus mitis	++
Streptococcus salivarius	++
Streptococcus mutans	++
Enterococcus faecalis	+
Streptococcus pneumoniae	+
Streptococcus pyogenes	+
Neisseria sp.	+
Actinomycetes	+
Spirochetes	++
Lactobacillus sp.	++

+ = common ++ = nearly 100 percent

2.2 Lab Material

2.2.1 Disposable Instruments

- Cyrovials, Nunc Cryotube Vials, Nunc Brand Products, Denmark.
- Culture flasks, 25 and 75 cm², Greiner Bio-one GmbH, Germany.
- Disposable needles size 4/12G, 1.5/20G, Braun, Germany.
- Disposable syringe size 10 and 20 ml, Braun, Germany.
- Eppendorf Cups 1000µl, disposable eppendorf pipettes tips, 100, 500, 1000, 2500 µl.

- Glass slides 76 x 26 mm, Mantel, #01/002 and 01/003, ResoLab, Bad Oeynhausen.
- Cover glasses, 18 x 18 mm, Menzel, #01/13, ResoLab, Bad Oeynhausen, Germany.
- P-B Test (Sarstedt) tubes 13 ml, graduated tube 50 ml, Greiner Bio-one GmbH, Germany.
- Haematocrite capillary L 75mm inner Ø1.1-1.2 mm, outer Ø 1.5-1.6 mm 20µl
- Non-pyogenic sterial filter, pore size 0.45µm, minisart / sartorius AG, Hannover.
- Non-pyogenic hydrophilic filter; pore size 0.20µm, sartorius, Göttingen Germany.
- Surfactant-free cellulose acetate filters 0.2µm pore size sartolab/ p20. /Sartorius AG, Göttingen Germany.
- ORIBRUSH, cell collector with protective tip B001, Orifice Medical AB, Malmo, Sweden.

2.2.2 Medium and Chemical Reagents

2.2.2.1 Medium

- DMEM (Dulbecco's Modified Eagle's Medium), Hepes modification (D6171) from Sigma plus supplements.
  - FCS 5 % (Fetal calf serum) kraeber GMBH and CO.
  - L-Glutamine (sterilized by filtration) Gibco, Scotland/ 15Q0174.
  - Gentamycine sulphate 80mg (Refobacin), Merck pharma GmbH, Darmstadt Germany.
  - Ciprobay 100 (ciprofloxacin), Bayer Vital, Leverkusen Germany.
  - Fortum (Ceftazidim 1.0g) cephalosporin, natiopharm GmbH, Ulm Germany.
- DMEM, (Dulbecco's Modified Eagle's Medium) from Gibco, (GlutaMAX+4.5g/glucose,-Pyruvate) plus supplements,
  - Hepes 30 mg/100ml, PH 7.5.
  - FCS 10 % (Fetal calf serum) kraeber GMBH and CO.
  - Gentamycine sulphate (Refobacin).
  - Glutamine sterilized by filtration
  - Ciprobay 100 (ciprofloxacin), Bayer Vital, Leverkusen Germany.
- DMEM (Dulbecco's Modified Eagle's Medium) from Sigma, powdered media with L-glutamine and 1000 mg/L glucose, without phenol red and sodium bicarbonate; D-2902, [steinheim Germany].

2.2.2.2 Chemical Reagents

- Hepes N-2-hydroxyethylpiperacine-N´-2-ethansulfonic acid (25Mm), powder.
- L-glutamine 200 mM, 5.8mg/200 ml, Gibco, Scotland.
- Trypsine (1:250 2.5%), stored at -20°C.
- EDTA (ethylenediaminetetraacetic acid).
- PBS (Phosphate Buffered Solution).
- 5-Aminolaevulinic acid (5-ALA), Fluka Chime GmbH clt-9471, sigma-Aldrich, Steinheim-Germany.
- Acridinorange / Ethidiumbromide.
- Chlorhexidine (D-gluconate) Forte 0.2%, 0.2 gm/ 100ml, GlaxoSmithKline Consumer Healthcare, GmbH & Co.KG, Bühl, Germany.

2.3 Equipment, Devices and Instrumentents

- Incubator, model 600 Memmert GmbH+CO.KG, Schwabach, Germany.
- Water bath incubator, GFL mbH type 1083, Burgwedel, Germany.
- Centrifuge, Megafuge 1.0 (fig. 2.1), Heraeus Sepatech GmbH, Osterode, Germany.
- Microhaematocrit centrifuge (fig. 2.2), HAWKSLEY, Gelman instruments, England.

(fig. 2.1)

(fig. 2.2)

- KNF Flodos Pump SM 18059, Sartorius, Göttingen, Germany.
- Fluorescent microscope, Ernst Leitz GmbH Wetzlar, D63227, Germany.
- Inverted microscope, Ernst Leitz GmbH, Wetzlar, Germany.
- Microscope camera (Wild-Leitz MPS 45) and TV recorder (Hitachi M235E VHS).
- Light microscope, Nikon Eclipse E 200.
- ThermoMix 1420, B, Braun, Germany.
- Stirrer, Janke & Kunkel GmbH & CO.KG, D7813, Germany.
- Vibrator, Janke & Kunkel, typ IKA-VIBRAX-VXR, Germany.
- Agitator, DESAGA Sarstedt- group SM1 Nr.18 02 00, GmbH, Heidelburg, Germany.
- Eppendorf pipettes, 10 µl, 100 µl, 500µl, 1000 and 2500µl. Eppendorf, Hamburg, Germany.
- Autoclave, Fritz Gössner. GmbH, Hamburg, Germany.
- Dryer, model 600 Memmert GmbH+CO.KG, Schwabach, Germany.
- PH measurement, inolab PH Level # 01100094, Webers GmbH.
- Optical probe. GRB light measurements.
- Fibermicroprobe –microspectrometer system.
- Digital camera, Nikon 5.0 mega pixels, Germany.
- Analytic balance, Mettler AT-261, Delta Range, Switzerland.
- Analytic balance, Mettler P2000 Switzerland.
- Sterile bench, Slee London, UK.

2.4 Methods

2.4.1 Preparation of cell culture media

2.4.1.1 Preparation of OAT cells growth medium,

- 500 ml DMEM Dulbecco's Modified Eagle's Medium, Hepes modification.

Supplements	
Activated FCS 5%	25 ml
L- glutamine	5.0 ml
Refobacin	0.5 ml
Ciprobay	2.5 ml
Fortum (Ceftazidim)	1.0 ml

The ingredients of the supplements mixed with medium stored at 4°C.

2.4.1.2 Preparation of SW1710 cells growth medium,

- 500 ml DMEM from Gibco (GlutaMAX+4.5g /glucose without Pyruvate)

Supplements	
Hepes PH 7.5	10 ml
Activated FCS 10%	25 ml
L- glutamine	5.0 ml
Refobacin	0.5 ml
Ciprobay	1.0 ml

The ingredients of the supplements mixed with medium stored at 4°C.

2.4.1.3 Preparation of freezing media

freezing media A

40 ml (10 ml) Fetal calf serum (FCS).
60 ml (15 ml) medium without supplement.

Freezing media B

20 ml (5 ml) Dimethylsulfoxid (DMSO).
80 ml (20 ml) medium without supplement.

The media stored at 4°C.

2.4.1.4 Preparation of medium without indicator,

For 5000 ml

Supplements	weighted by Analytic Balance, Mettler P2000
Distilled water(90% of final volume)	4950 ml
Sodium bicarbonate	3.7g/1000ml

The Mixture is stirred until dissolved. The pH of the medium is adjusted to 0.1-0.3 pH units below the desired pH (7.25). additional water is added to bring the solution to final volume. Sterilized by filtration filter 0.2µm pore size, stored at 4°C.

2.4.2 Preparation of chemical reagents,

2.4.2.1 Preparation of PBS (Phosphate Buffered Solution),

5 litres Distilled water

Ingredients	weighted by Analytic Balance, Mettler P2000
Sodium chloride (NaCl)	40 g
Glucose	4.5 g
di-sodiumhydrogenphosphate (Na_2Hpo_4)	6.5 g
potassium chloride (KCl)	2.0 g
Phenol Red (±)	2.5 ml

the ingredients added to water on stirrer, adjustment of pH 7.35 by 32% HCl and 5% NaOH, sterilized by filtration filter 0.22µm pore size, stored at 4°C.

2.4.2.2 Preparation of PBS Trypsine/EDTA,

PBS (with indicator)	200ml
Trypsine	5ml
EDTA	2ml

The solution sterilized by filter 0.45µm pore size and stored at 4°C.

- EDTA solution: 3.7g EDTA in 100ml alkaline bi-distilled water sterilized by filtration and stored at 4°C.
- Trypsine (1:250 2.5%). Stored at -20°C.

2.4.2.3 Preparation of Acridinorange / Ethidiumbromide,

- Stock solution

  49 ml distilled water + 1ml 95% ethanol.

  50 mg Ethidiumbromide

  15 mg Acridinorange

  Stored in deep freeze at -20°C.

- Work solution

1ml Stock solution
100ml PBS(with indicator)

stored at 4°C.

2.4.2.4 Preparation of 5-Aminolaevulinic Acid (5-ALA),

36mMol ALA stock solution	
5-aminolevulinicacid	60mg
PBS(without indicator)	10ml

dissolved the 5-ALA powder (weighted by Analytic Balance Mettler AT 261) in PBS, carefully adjusted the pH of ALA (5.0-5.5 pH) by 5 %NaOH due to the instability of 5-ALA [59]; sterilized by filter 0.20µm pore size and stored in dark at -20°C [60].

2.4.3 Preparation of cell cultures

2..4.3.1 Cryopreservation of cells

Tumor cell lines and HEK 293 cell line were frozen at concentrations between 5×106 and 10×106 /ml in 2 ml volumes. Pelleted cells were first resuspended in an appropriate volume of FCS medium A. The same volume of pre-cooled 20% DMSO(Dimethyl Sulfoxid) medium B was then gradually added to the cell suspension. After thorough mixing, 1.8 ml or 2 ml were transferred into freezing vials. The freezing vials were placed into freezing boxes and stored at –80°C.

2.4.3.2 Thawing of frozen malignant cell lines

The thawing media used for OAT cells, SW1710 cell line thawing was Dulbecco's Modified Eagle's Medium (DMEM), Hepes modification from Sigma and DMED from the company Gibco respectively. Tumor cell lines were frozen at concentration 5-10×106/ 1ml in 1ml volume, Thawed rapidly in a water bath at 37°C, as soon as only a small ice crystal was seen floating inside the freezing vial, the contents of a vial were transferred into sterial test tube, by additional 2 ml of growth medium washed out the freezing vial, immediately centrifuged at 1000 rpm for 3 min. Pelleted cells were then gently re-suspended in 3ml fresh growth media, and then transferred to 3 ml media in culture flask 25 cm^2. The cells are incubated at 37°C.

2.4.3.3 Cultivation of malignant cell lines and HEK 293 cell line

OAT cells are incubated at 37°C ; which are treated by approach based on the tendency of cells to growth and adhere to ground of Culture flasks, 75cm^2 (Greiner Bio-one) and grown in 50 ml medium with L-glutamine, supplemented with 10% heat–inactivated FCS, Gentamycine, Ciprobay, Fortum for 72 hours (minimum) 96 hours (maximum). For SW1710 (Urinary bladder carcinoma) the Dulbecco's Modified Eagle's medium is used, treated by 25 mM Hepes, 10% inactivated calf serum, 0.1 M glutamine, and antibiotics. For HEK Cells (human embryonic kidney) the Dulbecco's MEM treated with 10% inactivated calf serum was used.

2.4.3.4 Harvesting of cell lines

OAT, SW1710 tumour cell lines and 293 cell line were cultured in the standard tumour medium. The approach based on the tendency of cells to growth and adhere to ground of flask and grown in media. The volume of the medium in culture flask (75 cm^2 adherence surface) was 50 ml. every 3-5 days cells grew to confluence. The medium was removed, cells were washed for 10 minutes with 50ml Phosphate Buffered Solution (PBS) at room temperature, aspirate and discard. Directly trypsinized the cells by add of 5 ml of sterile prewarmed Trypsine-EDTA solution, incubated in the incubator (600/Memmert) and rocked the flask for 2-3 minutes to detach adherent cells. 293 cells can be detached by EDTA or tapping the flask by palm. Once dislodged, transferred the cell suspension to 5 ml growth media in graduated tube 50 ml (Greiner Bio-one), centrifuged for 3 minutes by 1000 rpm(Megafuge 1.0/Heraeus). The supernatant was discarded and gently resuspended the pellet with certain amount of media.

2.4.3.5 Cell counting and vitality test

Small cell culture flask (25cm^2 Greiner Bio-one) was filled with 6 ml medium and 500 µl cell suspension for cell counting. After 10 minutes The counting started on inverted microscope (inverted microscope, Ernst Leitz GmbH, Wetzlar), by magnification 32-lens objective. Nine counting squares of 150x150 µm/ culture were recorded, the average of counted cells was calculated. By factor 1.1x10^{5}x 2 we define the cell counting / 1ml medium. Fig 2.3 shown the formulas that have been used to calculate cell concentrations and the total cell numbers. The vitality test followed the counting by centrifugation of cells in a sarstedt (test) tube (13ml) at

1000 rpm for 3 minutes. The supernatant was wasted, and resuspended the cell pellet by 100µl of Acridinorange (cytoplasm staining) / Ethidiumbromide (DNA, nucleus staining).

cell concentration/ ml = average number of cells per square × $1.1\times10^{5}\times2$

total cell number = cell concentration × volume of cell suspension

Figure 2.3 Formula used to calculate cell concentrations and total cell numbers

15 µl (one drop) of cells was transferred to glass slide, covered and watched under water immersed lens (power 50) of fluorescence microscope (Ernst Leitz GmbH, wetzlar); excited by blue light. The stained cells were counted, about 200 cells to distinguish between the vital (green stained) and the non-vital (red stained). The number of vital/ total number of cells = % vitality.

2.4.4 Isolation of oral epithelial cells and saliva collection

2.4.4.1 Volunteers

From 37 volunteers with different oral hygiene and ages (18-45) years old, 50 brush biopsies have been prepared. The oral hygiene level of volunteers was in between score 0- score 2**,** according to World Health Organization (WHO) index [6]. Fourteen volunteers were score 0, fifteen volunteers were score 1, eight volunteers score 2. In addition to different concentrations of bacteria according to oral situation of oral cavity such as tooth or root caries, gingivitis, periodontitis.

2.4.4.2 Collection of saliva

The volunteers spat out 2 ml inside the test tube, and avoid the sucking of saliva. For 500µl saliva the same volume of DMEM Dulbecco's Modified Eagle's Medium from Sigma, without Phenol Red was added. in 4 Eppendorf cups (1ml). The saliva incubated in water bath incubator (GFL mbH type) at 37°C for three hours. The samples were centrifuged followed by Spectrophotometric examination to evaluate the values of autofluorescence photodetection of oral flora that grows during the incubation time, that have the ability to produce and release PpIX, without any exogenous of 5-ALA.

2.4.4.3 Oral epithelial cells

By small smooth brush we had isolated epithelial cells from dorsum of tongue, buccal (check) or attached gingiva (fig. 2.4). The ORIBRUSH bristled brush rotated 5-10x in one spot , to access and sample all epithelial layer [61]. The cellular material collected on the brush is directly immersed in (Sarstedt) tube with 3 ml DMEM Dulbecco's Modified Eagle's medium from sigma (without phenol red), and centrifuged for three minutes, at 1000 rpm. The brush is removed gently and wasted. The supernatant and pellet resuspended very well. 3 eppendorf cups with 3 ml suspension have incubated for 3 hours in water bath incubator at 37°C.

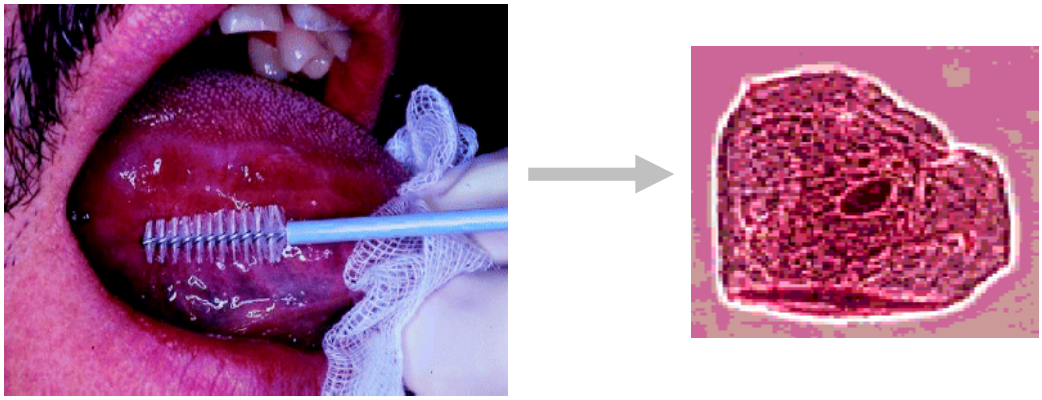


fig. 2.4. Isolation of oral epithelial cells by ORIBRUSH from lateral of healthy tongue (right), the oral epithelial cell (left) [61].

2.5 Experimental setup used for autofluorescence measurements

2.5.1 Microoptical probe

The RGB detector, has light emitting diode (LED). An is used to excite the photosensitive PpIX in the cells by filtered blue light (405 nm wavelength). The light transported through bundle of fibers (fig. 2.5). The distal tip of the microoptical probe fibre; has been inserted in fine tubes or Eppendorf cups completely or with a distance of 1 mm to the pellet. The prepared cell samples that were treated by ALA, absorb the blue light, and emit the red fluorescence. The detector measured and registered the intensity value of three color zones (red > 590 nm; green 510-590 nm; blue 450-510 nm).

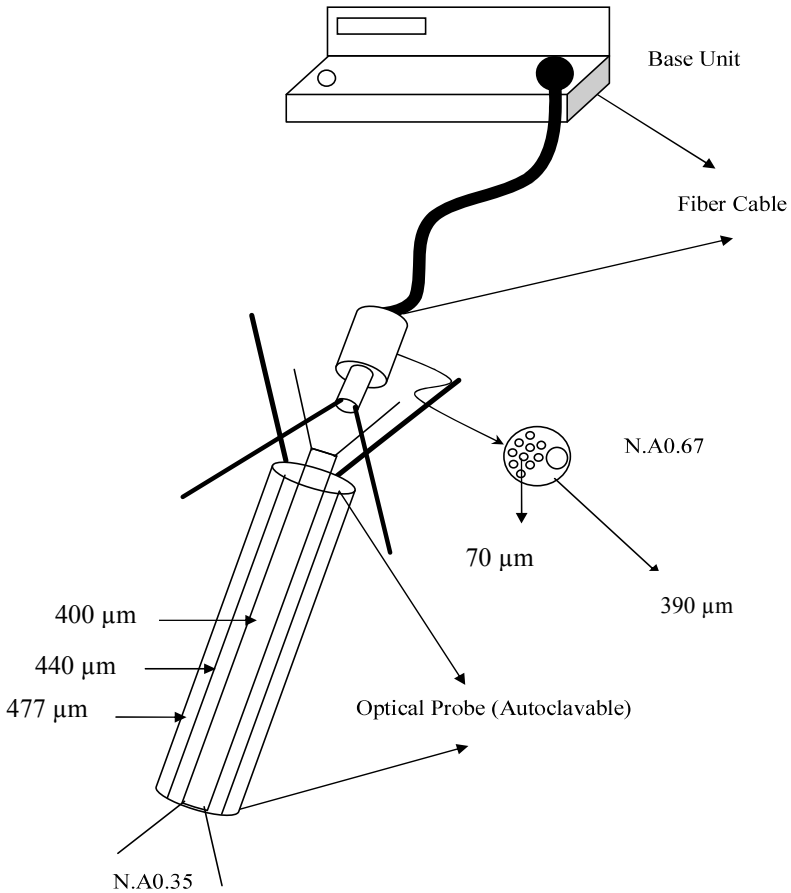


Fig. 2.5. microoptical probe (RGB measurements)

2.5.2 Spectrofluorometer

Cells fluorescence spectra were obtained using the measurement set-up shown in (small spectrofluorophotometer) Fig. 2.6. The excitation wavelength was 405 nm blue light, and measuring the red fluorescence by 635 nm [62] to detect PpIX. The total output at the tip of the probe was about 370 µW. Fluorescence spectra were recorded in the 450 800 nm range, with time of integration 2560 ms. The filter (>455 nm) was placed in front of spectrometer, to prevent any scattered excitation light from reaching the detector. As well removed any reflected excitation light. The distal end of the fibre was placed in contact with the cells pellet in eppendorf cups, in capillaries with distance of 0.25-0.50 µm to be examined. the excitation light radiates in 15°. Fluorescence intensities were registered at about 635 nm and 700 nm from tumor [48]. (maximum peak of PpIX fluorescence). Fluorescence intensities values were calculated automatically in EXCEL.

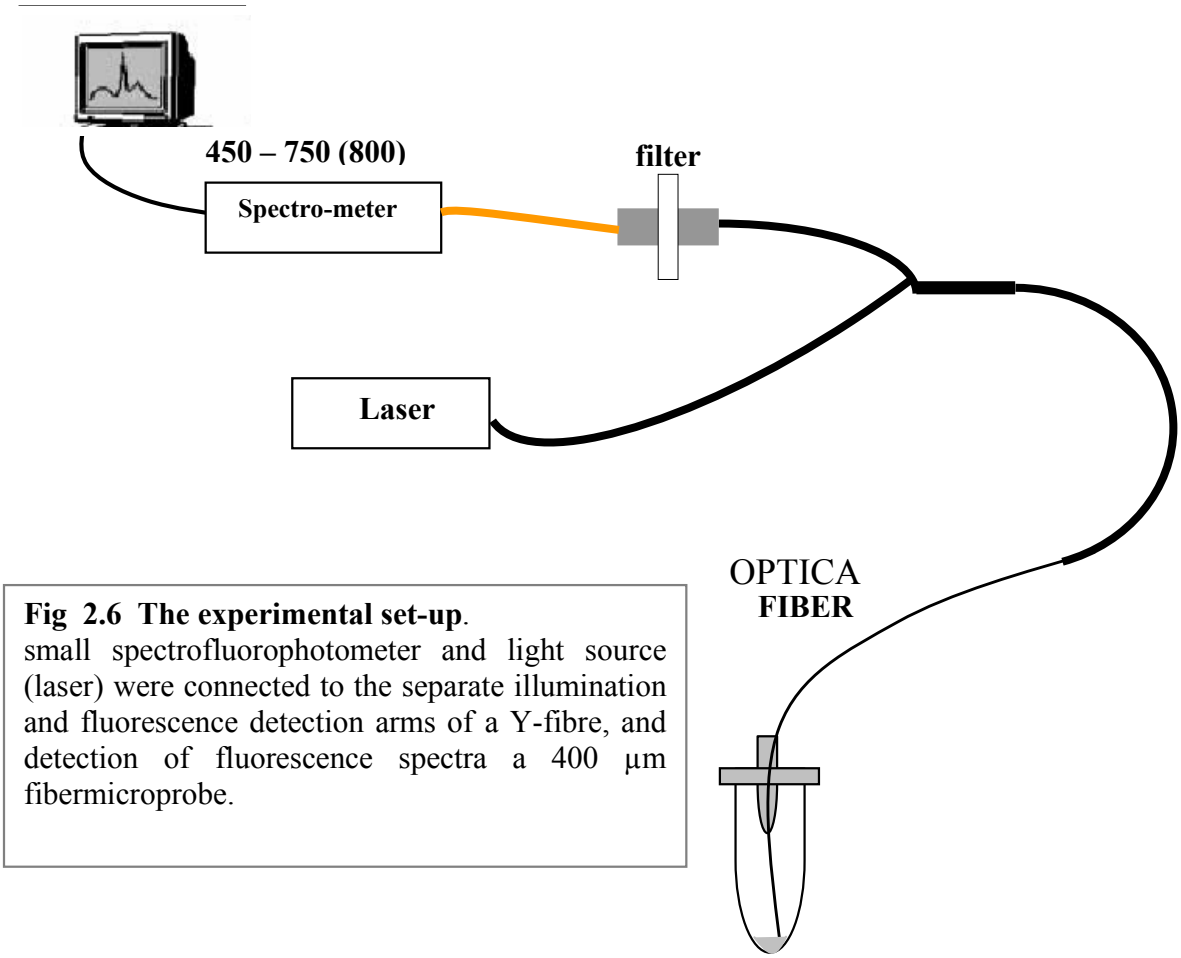


Fig 2.6 The experimental set-up.
small spectrofluorophotometer and light source (laser) were connected to the separate illumination and fluorescence detection arms of a Y-fibre, and detection of fluorescence spectra a 400 µm fibermicroprobe.

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2.6 Preparation of experimental samples

Samples with different cell concentrations were prepared by different methods of dilution. More than 50 samples incubated with 5-ALA-inducing endogenous PpIX (5-ALA/Fulka), for three hours. 100, 500 tumor cells as a final concentration in Haematocrite capillary and Eppendorf cups. Four million cells were incubated for three hours on Agitator (DESAGA, Sarstedt- group SM1); in incubator (Memmert model 600), after application of 5-Aminolevulinic acid at a final concentration of 2mMol (55.5 µl/1ml) and ph 5.5. followed that, and for investigation lower concentration of cells, the samples prepared by re-suspension of cells by appropriate volume PBS without indicator. We used haematocrite tubes to infuse the cell suspension then closed them by torch melting (Fig. 2.7). furthermore, one and two million cells were diluted with dilution medium without indicator. 250 µl cells suspension / 50 ml media without indicator, resuspended in graduated tube 50 ml, Greiner Bio-one GmbH (Fig 2.8). In eppendorf cups 100 cells/ml incubated with 2mM 5-ALA (Fulka) in the dark at 37°C for three hours.

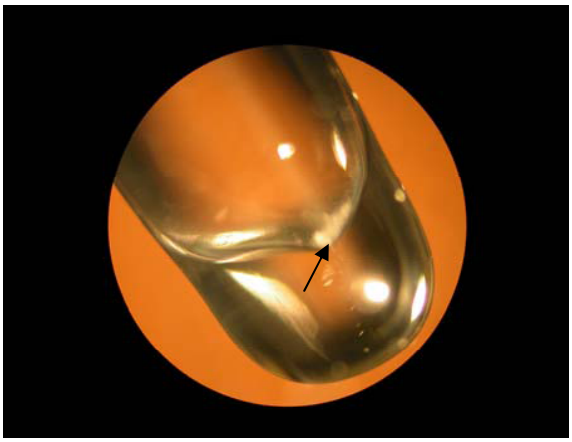
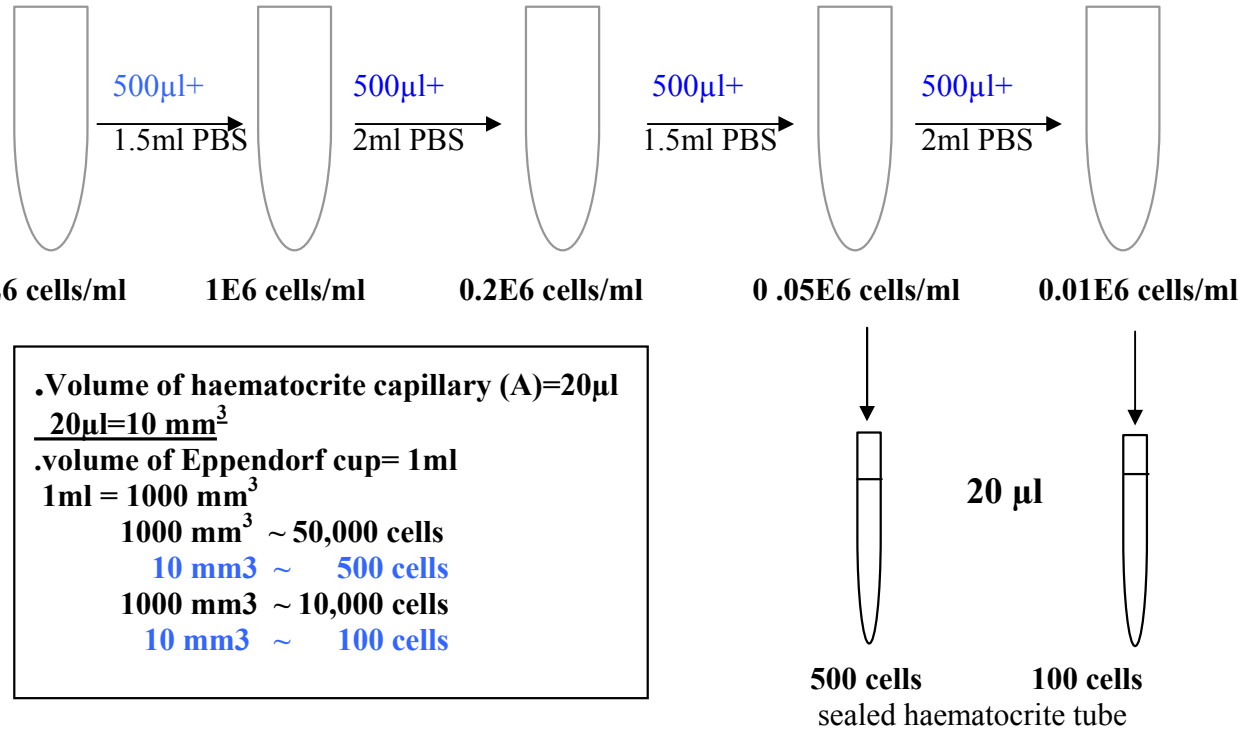


Fig 2.7 sealed haematocrite tube on melting, 20x enlarged (Light microscope, Nikon E200), (arrow) cells pellet of OAT cells after centrifuged 3 minutes in microhaematocrit centrifuge HAWKSLEY instruments.

2.7.1 Serial dilution of cells with PBS

The serial dilution is (simply) a series of simple dilution, the source of dilution material for each step comes from the diluted material of the previous [63]. 4 million cells/ml as a control and 5-ALA treated samples were incubated in eppendorf cups for three hours. From 4E6 sample, different cells concentration samples have been prepared by dilution and re-suspension of cells by different volumes of PBS, followed by infusion of the cells suspension in haematocrite tubes, sealed on melting (fig 2.8).



More than 50 samples of OAT and SW1710 cells, were prepared by serial dilution; and used to investigate 100 and 500 cells as a final concentration of cells / 20µl by optical probe and spectrometer.

2.7.2 Simple dilution of cells with media

The dilution of 1E6 and 2E6 tumor cells with a ratio 1:200, by resuspended of 250µl of original concentration of cell suspension in 50 ml standard tumour medium. Followed by cell counting 40 ml cell suspension in small cell flask. Under 10er lens objective of inverted microscope, about 2 cells/ 150µm x 150µm, the cell concentration per ml media was defined. Fig 2.8 gives the calculation of final cell concentration in Haematocrite tube (20 µl).

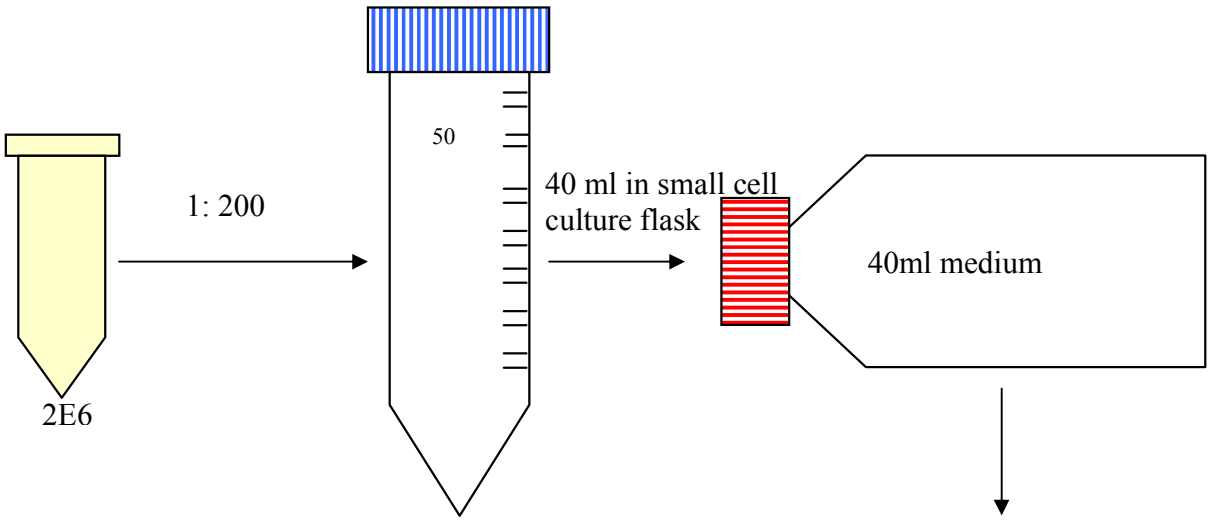


Fig. 2.8 Dilution and Calculation 2 million cells.

2.7.3 Preparation of brush and saliva samples

2.7.3.1 Isolation of cells by brush from cell culture (ex-vivo biopsy)

The brush samples performed by isolation of tumor cells from 1E6 cell culture. as in vitro biopsy. The brush was inserted into cell culture; gently touched the monolayer cells, that grow attached to the ground of culture flask. Directly embedded the brush in Dulbecco's Modified Eagle's medium from sigma. Centrifuged for 3 minutes, 1000 rpm. The brush was removed

gently and wasted, the supernatant and pellet are resuspended very well. The suspension was transferred to small cell culture flask (25cm^2 Greiner Bio-one) fig 2.9. The counting was started under microscope camera (wild-leitz MPS 45) by magnification 10 lens objective. By factor 1.1x10^4 we define the cell counting / 1ml medium, before starting the incubation of 1ml samples with 5-ALA.

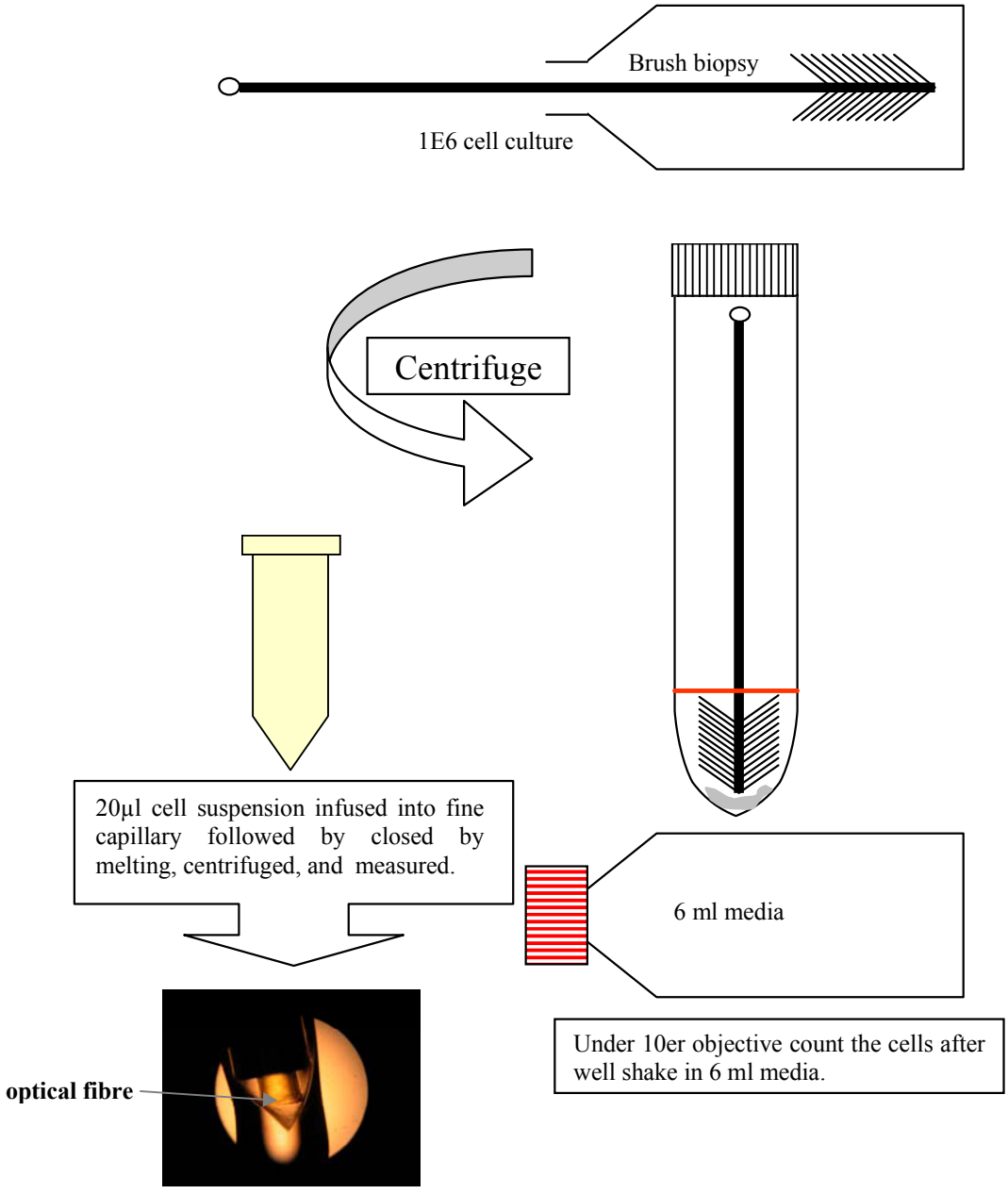


Fig 2.9 Isolated Cells by brush from Cell culture 1E6

Many samples were prepared from brush isolated cells in eppendorf cups, and treated with 5-ALA at a final concentration of 2mMol (55.5 µl/1ml) and ph 5.5. The samples incubated for

three hours, at 37°C on Agitator (DESAGA, Sarstedt group); in incubator (Memmert model 600). 20 µl were transferred to the haematocrite by infusion and centrifuged 1000 rpm for three minuets, followed by investigation of samples by a 400 µm fibermicroprobe – microspectrometer system.

2.7.3.2 Isolation of oral epithelial cell by brush (vivo biopsy)

The procedure was carried out by isolation of cells by brush from different oral mucosa of healthy volunteers. Followed by direct immersion of brush in DMEM without Phenol Red, and centrifuged. The samples (1 ml in eppendorf cup) were divided to three groups. First group (A), the samples were washed after incubation, to eliminate most of bacteria. Second group(B) of samples, the cells isolated, after instructed of volunteers to rinse the mouth by PBS and chlorhexidine 0.2% (0.4 mM/l). The third group(C), the samples have been incubated with 10 µl chlorhexidine 0.1%. all samples incubated with media without indicator (in vibratory water bath incubator GFL mbH type 1083). The incubation time was 2 hours in darkness at 37°C, after application of 2 Mm ALA (5.55µl), in the addition of samples free from 5-ALA served as controls. At the end of incubation, 1ml samples were (group A) centrifuged and washed once, twice, and three times with phosphate buffered solution (PBS) at pH 7.35 (without Phenol Red). Photosensitization was carried out by illumination of samples before and after washing. All samples from all volunteers (three groups) investigated by microspectrometer, and under fluorescent microscope stained, to define different concentration, kinds of microorganisms that attached to epithelial cells (Fig 2.10), and the number of epithelial cells in all samples.

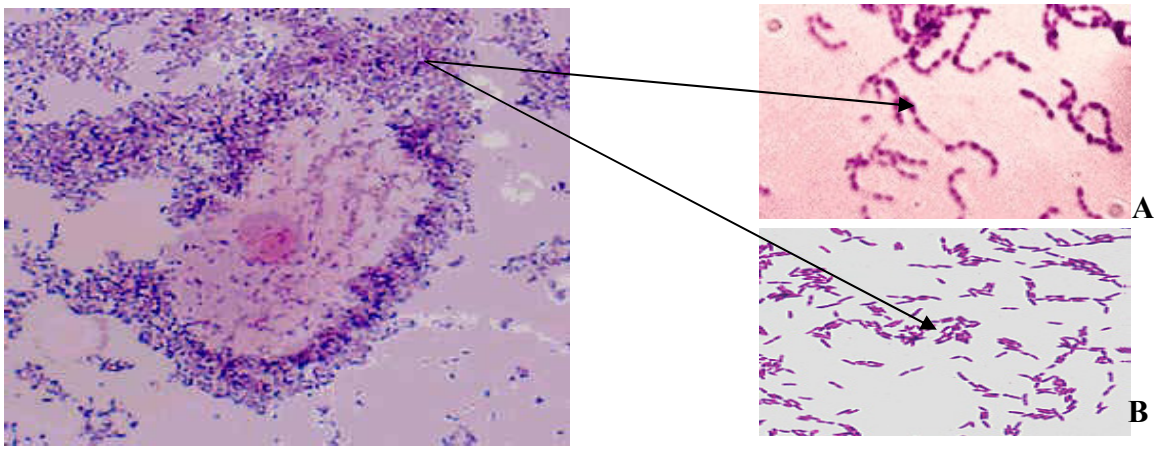


Fig 2.10. (a)oral epithelial cell, (b)oral flora attached to the cell surface [20].
(A) Streptococcus mutans. (B) Lactobacillus species.

The fluorescence intensities were registered at about 635 nm and 700 nm for epithelial cells and bacterial signal (control samples), as a result of production of PpIX, whereas the auto-fluorescence of cells registered at around 500 nm[48].

2.7.3.3 Preparation of saliva samples

The saliva collected from volunteers with different oral hygiene that, instructed to simply spat out 2ml of saliva (not to suck saliva) into tube (Sarstedt 13 ml). For every 500 µl saliva we added 500 µl media without phenol red, transferred to 4 eppendorf cups. 2 mMol final concentration 5-ALA was added to 3 cups and one served as a control. The 4 samples were incubated in the darkness in vibratory water incubator (GFL mbH type 1083) at 37°C for 3 hours. followed by centrifugation in (Megafuge) Heraeus for 3 min, 1000 rpm. The measurements started by microspectrometer; first the control and one 5-ALA sample (without washing). The other samples were washed with PBS and centrifuged. One sample was measured. the residual sample were rewashed again in PBS without indicator, centrifuged and measured by microspectrometer. Different fluorescence intensities measurements were registered in the range between 630 nm and 700 nm from bacteria to define the ability of bacteria to produce endogenous PpIX [64]. Saliva samples contain about 200 species of bacteria [20]. All saliva samples investigated; before and after washing under the fluorescence microscope to defined the amount of residual and eliminated bacteria.

2.8 Data Analysis

The fluorescence spectra were evaluated in this study, by LIGA-Microspectrometer system after beeing transferred to a personal computer, where the fluorescence read out every three seconds and calculated automatically in EXCEL (Fig. 2.11). The recorded measurements of different intensities from different cell lines normalized. The autofluorescence in the wave length range between 580 and 730 nm was approximated by fitting an exponential curve in the wavelength intervals between 530 and 580 nm, and between 750 and 800 nm, to each recorded spectrum [65]. After subtracting this background, the PpIX fluorescence was calculated at 635 nm (A). To evaluate the formation of photoproducts, the background-free intensity at 670 nm (B) was evaluated. The maximum intensity of cell auto-fluorescence at 460 nm (C) was assessed (Fig. 2.11). Spectral data from the healthy volunteers, were analyzed to determine the amount of variability in the fluorescence intensities of oral brush samples

from different locations, in the red region (635-nm). Peak fluorescence intensities from different sites (different kinds of bacteria) were compared with those from malignant cell lines. A similar comparison of measurements by microoptical probe was made using the averaged values of the fluorescence intensities, at the red, blue, and green regions, for controls and the 5-ALA samples of normal and malignant cell lines.

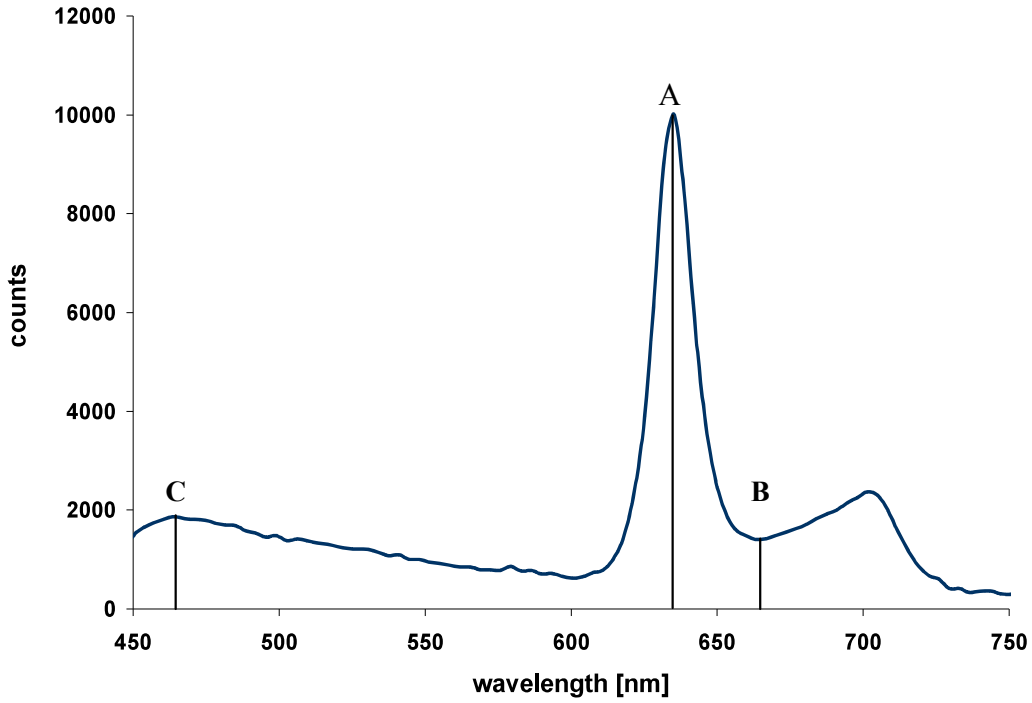


Fig. 2.11. The fluorescence intensities of 1E6 SW1710 cells, after incubation for three hours in (Memmert model 600) at 37°C, with 5-ALA at a final concentration of 2mMol (55.5 µl/1ml) and pH 5.5, centrifugation 1000 rpm ≈ 140 g.

2.9 Experiments Protocol Tables

Table 1. Cell cultures

	75 cm^2 Flask	**25 cm^2 Flask**	**Time (min)**
Aspirated medium	50 ml	6 ml	
Washing by PBS on the Agitator	50 ml	6 ml	10
Aspirated PBS, detachment of cells by Trypsine/EDTA	5 ml	3 ml	2-3
Quantity of media transported to cell suspension centrifuge tube	5 ml	3ml	
Flask washing with medium	5 ml	3 ml	
Centrifuge at 1000 U/min 140g			3
Resuspension of cells by medium	depends on cell pellet size	depends on cell pellet size	
Cell counting sample	500 µl cell suspension	Bring back to small flask 6 ml medium	
Centrifuge at 1000 U/min			3
The medium wasted and stained by Acridinorange/Ethidiumbromide	100 µl	100 µl	
Microscopic test (blue excitation)	10-15µl	10-15µl	

Table 2. Oral brush biopsy protocol

	(Sarstedt) tubes 13 ml	**Eppendorf cups 1 ml.**	**Time (min)**
Oral brush biopsy medium	4 ml		
Centrifuge at 1000 U/min 140g			3
Resuspension of pellet by centrifuged medium	The same medium		
Incubated oral samples		1 ml (4 cups)	180
Microspectrometer investigation		1 ml	
Centrifuge at 1000 U/min			3
The medium wasted and stained by Acridinorange/Ethidiumbromide		100 µl	
Microscopic test (blue excitation)		10-15µl	

Table 3. Saliva biopsy protocol

	(Sarstedt) tubes 13 ml	Eppendorf cups 1 ml	Time(min)
Spat out saliva	2 ml		
Quantity of media transported to saliva	2 ml		
Incubated saliva samples		1ml (4 cups)	180
Centrifuge at 1000 U/min			3
The medium wasted and pellet Washing by PBS		1 ml PBS	
Centrifuge at 1000 U/min			3
The medium wasted and stained by Acridinorange/Ethidiumbromide		100 µl	
Microscopic test (blue stimulation)		10-15µl	

Table 4. Incubation of samples with 36 mM 5-ALA-stock solution

	Eppendorf cup 1ml	Time (hours)
Control (1control)	1ml with 1E6, 2E6, 0.05E6 and 0.01E6 OAT, SW1710, 293 cell lines.	3
Samples (4 samples)	1ml with 1E6, 2E6, 0,05E6 and 0.01E6 OAT, SW1710, 293 cell lines + 2 mMol ALA (55.5 µl/ml)	3

Table 5. Preparation of 500, 100 cells microtubes samples

	Haematocrite capillaries 20µl	Time (min)
0.05E6/ml control	500cells/20 µl	
0.05E6/ml ALA sample	500cells/20 µl	
0.05E6/ml control	100cells/20 µl	
0.05E6/ml ALA sample	100cells/20 µl	
Centrifuge at 1000 U/min 140 g		3-5
Microspectrometer investigation	20 µl	

Table 6. Incubation of oral epithelial brush samples with chlorhexidine forte (0.4 mMol/l) and 36 mM 5-ALA-stock solution.

	Eppendorf cups	Time (hours)
Oral biopsy control	1 ml sample suspension + 10 µl chlohexidine Forte 0.1%	2-3
Oral biopsy control	1 ml sample suspension	2-3
Oral biopsy sample	1 ml sample suspension + 10 µl chlohexidine Forte 0.1% + 2 mMol 5-ALA (55.5 µl/ml)	2-3
Oral biopsy sample	1 ml sample suspension + 2 mMol 5-ALA (55.5 µl/ml)	2-3

- **Sterilization of instruments**

All the Lab instruments are sterilized by washing with detergent material, dried in dryer (Memmert model 600) at 70°C for 2 hours (Hot air sterilization), covered by foil. autoclaved at 132°C for 25-30 minutes, in steam autoclave (Fritz Gössner GmbH), followed by drying in dryer for 2-3 hours. Directly transferred to the Lab.

- **Sterilization of working bench**

The bench Sterilized by Incidur spray 96% Ethanol (Ecolab GmbH Wien) and over night UV light.

- **Sterilization chemical reagents**

By different sizes of pyogenic filters, all chemical reagents and media without indicator are sterilized by pump membrane filtration [Sarstedt, filter 0.2 µm, 0.45 µm]

- **Sterilization of cell culture medium**

The growth medium is not only convenient growth media for cells, even the microorganisms which considered as main reason to cell cultures infection. By broad spectrum antibiotics, the medium have sterilized before using. The antibiotics added by the same procedure as in 2.4.1.

- **Distillation of water**

The water that used to prepare chemical reagents like PBS must be distilled. The distillation performed by Seralpur (Reinst wasser system), water purification system.

3 Results

3.1 The small number cell samples

The present study has shown that small number cell samples can be achieved by dilution of large numbers cell samples, which is described in the material and methods section (2.7.1, 2.7.2). The ultimate goal of this reduction was to investigate the efficiency of the microspectrometer to detect the PpIX in limited number of different cells. We maintained the cells vitality for period of time as a non-adhesive culture bowl by using of eppendorf cups, after the incubation of about 50 samples in test tubes (13 ml) and eppendorf cups, the vitality test has shown that the eppendorf cups are more suitable to incubate tumor cell lines (OAT, SW1710) and HEK293 (fig 3.1).

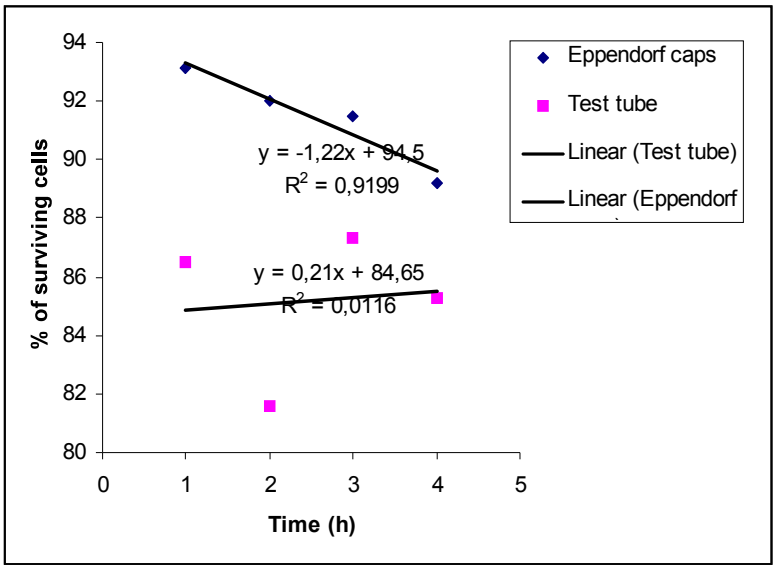


Fig. 3.1 30 Samples incubated in test tubes and eppendorf cups, the vitality test has shown, that the eppendorf cups are more convenient to incubate different cell lines than test tubes as a non-adhesive culture bowl for three hours.

In order to quantify and determine the small number of cells, we have counted the cells after dilution of 4E6/ml. The calculated numbers in 20µl (haematocrite tube volume) suspension are diluted by serial and simple dilution [63]. 50 samples have been counted, and the average number of every 10 samples are calculated as shown in the Table 3.1. The simple dilution of 1E6 and 2E6 of tumor cells with a ratio 1:200, followed by counting of cells in 40 ml cell suspension in small cell flask under 10er lens objective of the inverted microscope. The cells concentration per ml media have defined. Final cell concentration in 20 µl has been calculated according to the following approach:

Average number of cells * 11000 = number of cells(X) /40ml

X divide 40 ml = number of cells(Y) /ml

Y * 20 µl div 1000 = number of cells / 20 µl

Average Nr. of cell/20µl in 10 samples	Average 1	Average 2	Average 3	Average 4	Average 5	
Serial dilution of 4E6 cell/ml	123	144	106	110	137	124 ± 20
Simple dilution of 1E6cell/ml	56	62	51	47	67	56.6 ±10
Simple dilution of 2E6cell/ml	104	169	129	98	140	128 ± 18

Table 3.1 The average numbers of cell counting after serial and simple dilution of 50 samples 4E6, 1E6 and 2 E6 OAT and SW1710 cell/ml, respectively.

The experiments were carried out; in order to determine the appropriate concentration of cells, approved that simple dilution of 2E6 with a ratio 1:200 gives minimum concentration of cells that exhibits a significant peak after application of 5-ALA. Between 100-150 cell/ 20µl have been counted after simple dilution of 2E6/ml (fig 3.2).

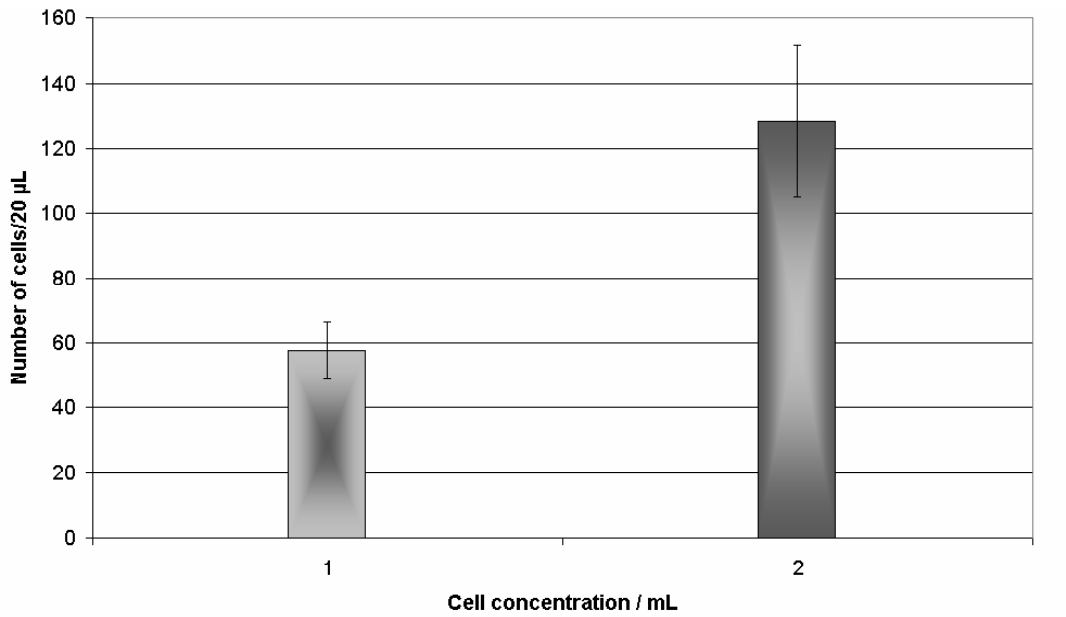


Fig 3.2 (1) Average number of cells in 20µl from 1E6 SW1710 cell /ml, (2) Average number of the cells in 20µl from 2E6 SW1710 cell /ml.

3.2 Kinetics of 5-ALA induced PpIX in small number cell samples

The minimum concentration of OAT, SW1710 and HEK293 is used to study the kinetics of 5-ALA-induced PpIX fluorescence in tumor cells and in normal cells. Fluorescence intensity has been taken as parameter for PpIX in the different cell lines. After application of 5-ALA at a final concentration of 2mM, preferential PpIX accumulation in the tumor cells have been detected [66]. Also, a constant increase with the time in PpIX fluorescence intensity in tumor cells [67], have been observed 3 hours after application of 5-ALA, in regardless of the concentration of the cells, the repeated measurements, 1 hour later did not show any increase in the fluorescence intensity. Three hours after 2mM application of 5-ALA was the optimal detection point [68]. The cells excitation wavelength was 405 nm (blue light), emitted the red fluorescence where PpIX shows maximum fluorescence at 635 nm (fig 3.3). After 4 hours, the height of peak starts decreasing and this is proved by the measurements, which have done by the low concentration of cells in the micro-tubes (haematocrite capillaries).

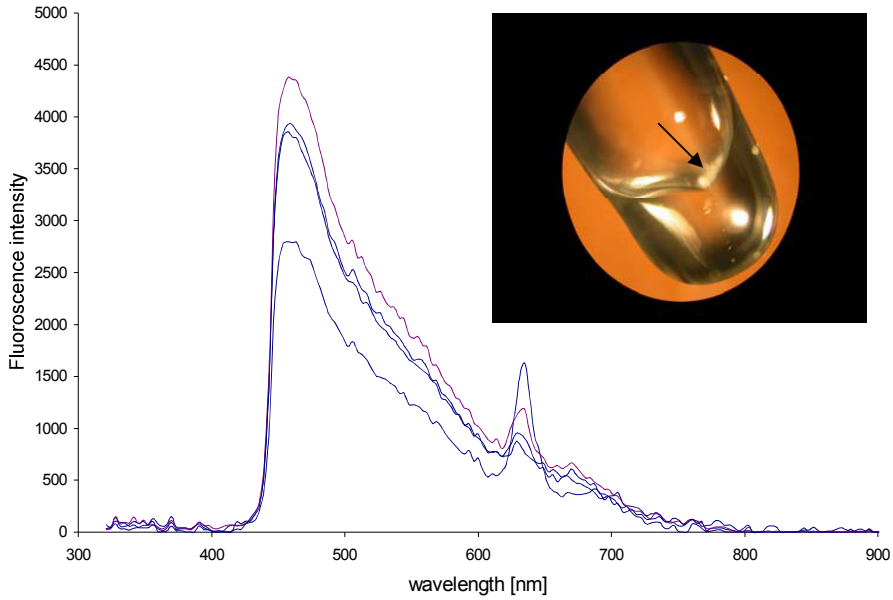


Fig 3.3 The fluorescence intensities for 100 OAT cells (2E6 cell/ml as a final concentration in eppendorf cups, 100 cells in haematocrite 20/µL) after three hours incubation with 2mM 5-ALA. Cell pellet (black arrow) centrifuged in microhaematocrit Centrifuge for 3 min (12,000 rpm) after being sealed on melting (the intensity decreased due to photobleaching).

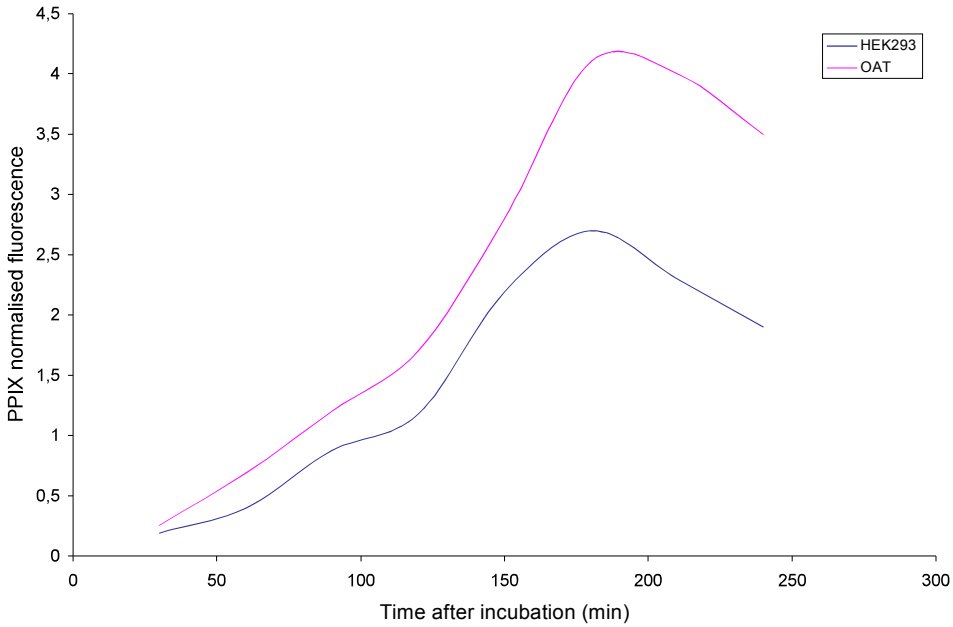


Fig 3.4 Pharmacokinetics of 5-ALA-induced PpIX fluorescence. A representative eight samples was incubated with 2 mM 5-ALA, centrifuged and the PpIX fluorescence intensities were recorded in tumor cells and normal cells HEK293.

As well as in 500 cells/20µl concentration (malignant or normal cell lines), three hours incubation time after application of 2mM 5-ALA was the optimal detection point of PpIX at 635 nm (fig. 3.5). The tumor cells tended to reach maximal PpIX fluorescence earlier than the HEK293 cells. On the other hand the maximum of PpIX fluorescence intensity in high concentration tumor and HEK293 cells is reached nearly at the same time.

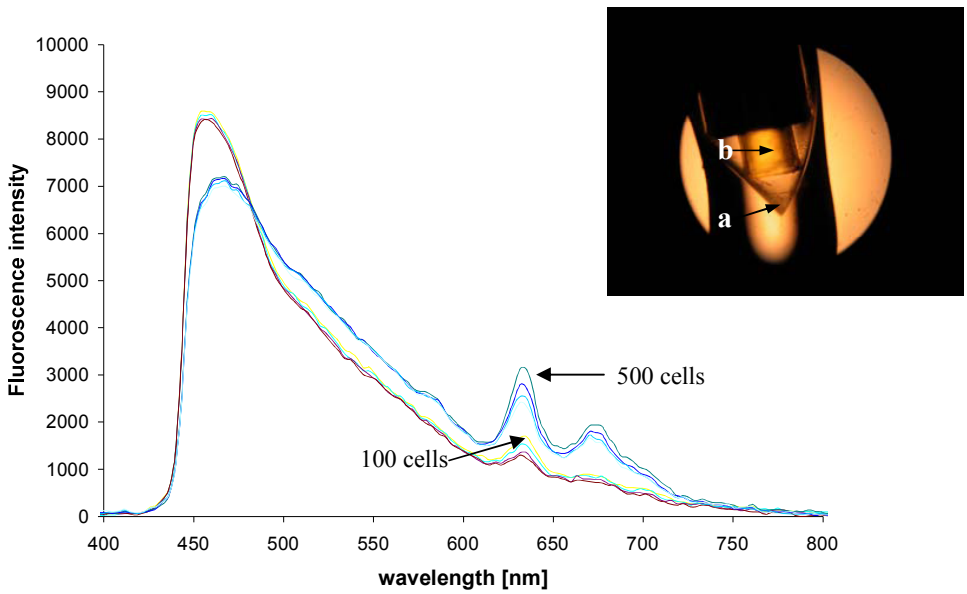


Fig 3.5 Fluorescence intensity of 100, 500 OAT cells (the graph) in haematocrite capillary after 3 hours incubation with 2 mM 5-ALA. The picture of sealed haematocrite tube (20x enlarged) was taken under light microscope, Nikon E200 (a) 500 cell pellet, (b) optical fiber.

3.3 Evaluation of kinetics of 5-ALA induced PpIX in 100, 500 cells by microoptical probe

More than 50 samples with different concentration of cells, have been treated by 2mM 5-ALA, incubated for 3 hours, followed by measurement with the microoptical probe. All the measurements of ´´minimum numbers of cells``, have been done in haematocrite capillaries, by insertion of the optical fibre into the capillary, that has been positioned in the fine tube rack. The microoptical probe recorded the fluorescence intensity of three colour channels (red, green, blue). The averaged values of 100 and 500 cells that were treated with 5-ALA and that served as a control were calculated, as well as the standard deviation (SD) of single measurements. Four samples, one served as a control; were measured after three hours incubation time. The standard deviation of single measurements of every colour have been calculated, SD of single measurement of 5-ALA treated sample (100 cells) is in the range of 1-4 % and 1-3 % SD for 500 cell sample (fig 3.6, 3.7).

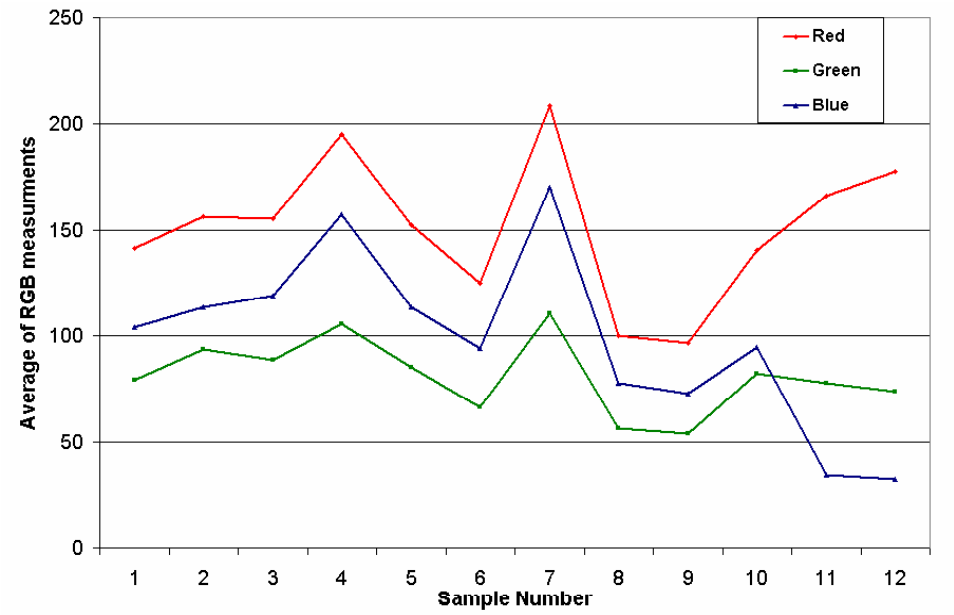


Fig 3.6 Averaged values of optical probe measurement for 100 OAT cells with 5-ALA, after three hours incubation, standard deviation (SD) of single measurement 1-4 %

Figure 3.8 shows the same data of averaged values of fluorescence measurements of 5-ALA samples, that were recorded at 405-nm excitation, by using of microoptical probe in comparison with controls. The fluorescence intensity measurements at red region is greatly varied between the 5-ALA samples and control. The lowest total intensities were recorded for 100 cells that served as control. The increasing of fluorescence of PpIX in the tumor cells depends on the concentration of cells (the number of cells / 20 µl).

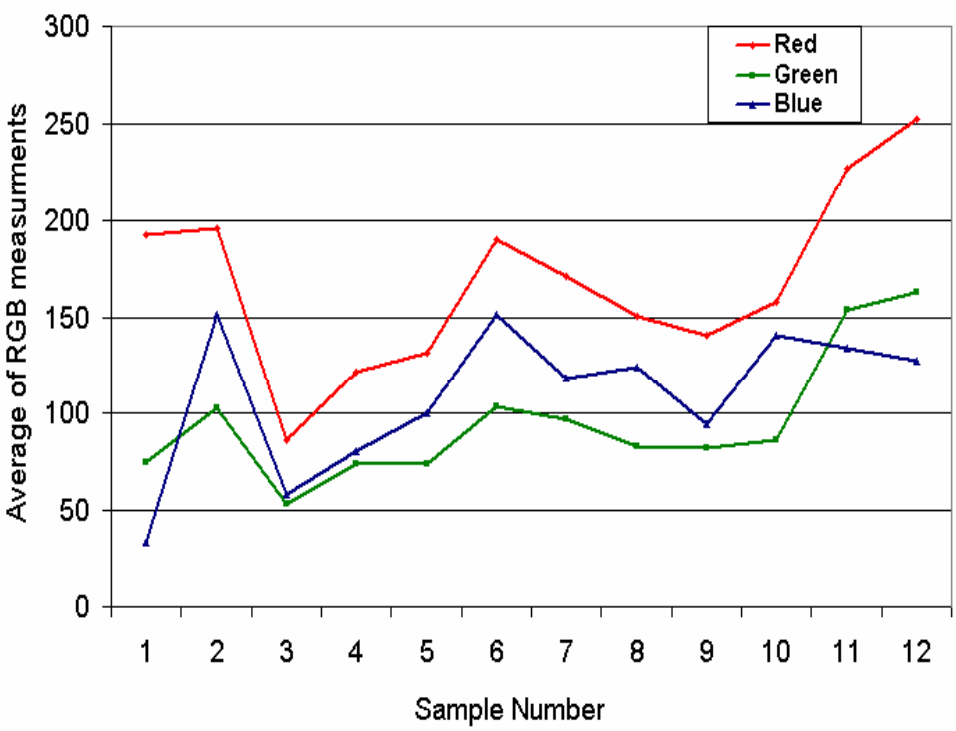


Fig 3.7 Averaged values of optical probe measurments for 500 OAT cells with 5-ALA, SD of single measurment 1-3 % (after three hours incubation).

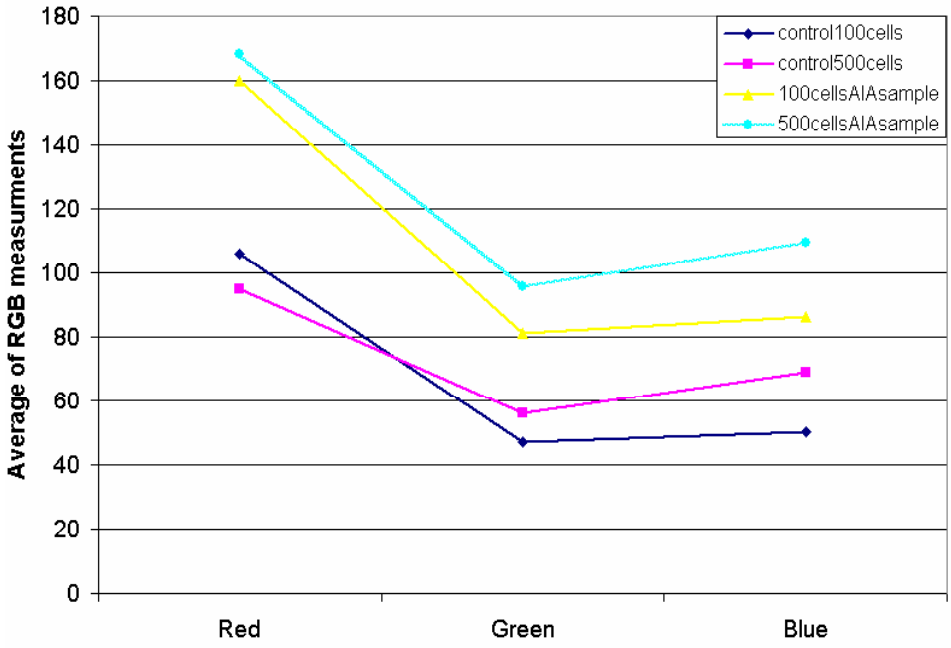


Fig 3.8 Fluorescence analysis of averaged values of microoptical probe measurement (RGB) 100 and 500 cells control and 5-ALA samples (after three hours incubation).

After three hours incubation and from the data of the three colour channels, that have been detected at 405-nm excitation, the data revealed significant difference between controls and 5-ALA treated samples with limited cell numbers.

3.4 Amount of malignant and 293 cells in ex-vivo brush samples

In common with brush biopsy, that are commonly used as sampling technique for lung, nasal tissue collection and in gynaecology [69], we have used the brush to collect cells from culture flask. Cultures with 1E6 cells have been prepared, followed by insertion of stiff bristled brush into the culture. By brush the monolayer of growing cells have gently touched, centrifuged and counted to determine the number of cells that have been collected by brush (table 3.2). Many samples from different cell lines (in 6 ml media) were counted under 10er objective (factor $1.1x10^4$). Between 4E3-10E3 / ml, the amount of cells that could be collected by gentle touch of cell layer (fig 3.9).

Table 3.2 Averaged number of cells in 150 x 150 µm under 10er objective, concentration of cells per ml that were collected by brush from 1E6 cell culture.

Sample Nr.	1	2	3	4	5
Average Nr. of 10 samples	4.2	7.7	8.4	4.4	5.7
Concentration per ml	4.7×10^3 cells	8.5×10^3 cells	9.2×10^3 cells	4.8×10^3 cells	6.3×10^3 cells

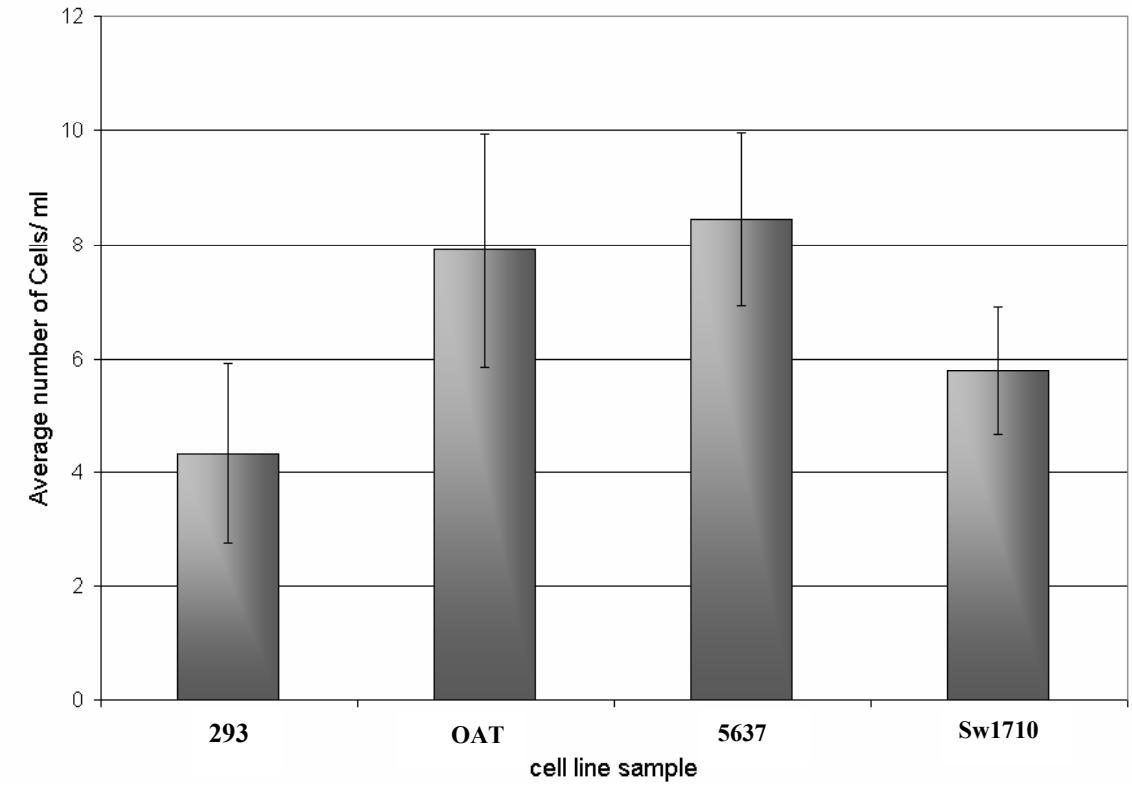


Fig 3.9 Averaged number of different cell lines per ml (4E3-9E3) that were collected by brush from 1E6 cell culture. Standard deviation is given for all samples 1-2%.

In eppendorf cups 30 samples were incubated in the darkness at 37°C after application of 5-ALA. Different concentration were prepared from brush biopsy, incubated for three hours, followed by transfer a limited number of cells, into 20 µl capillary.

In regardless of the concentration of cells, OAT cells revealed the highest values of PpIX fluorescence at 635 nm after three hours incubation with 5-ALA. There is significant difference between maximum PpIX fluorescence intensities in 1E3 cell / 20µl malignant cell (OAT) and HEK293 as normal cell line (Fig 3.10). The ratio was approximately 10:1 (after normalized to 460 nm peak).

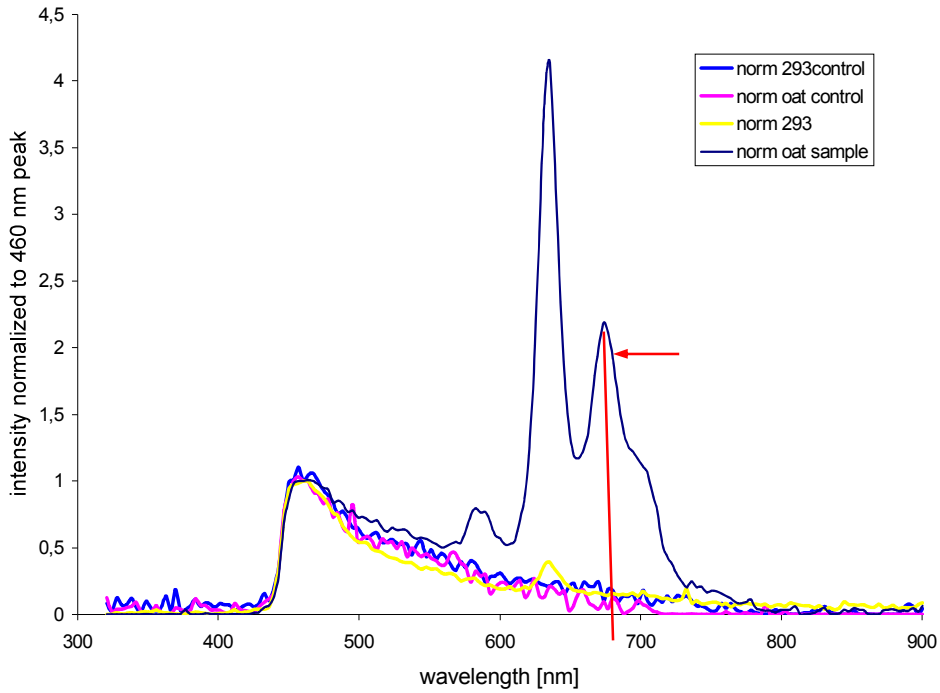


Fig 3.10 Typical normalized (at 460 nm) fluorescence spectra for 2 mM 5-ALA treated OAT and 293 cell lines after 3 hours incubation at 405 nm excitation. The build up of dual–peaked PpIX and photoporphyrin fluorescence in red wavelength region (653 and 673 nm).

The control samples have the same fluorescence signal in the different wavelength regions for both cell lines. The experiments showed that the fluorescence intensities of control samples are at least 100% less than the ALA samples at red wavelengths. Fluorescence intensity that has obtained from SW1710, and in comparison to OAT cells; they revealed less fluorescence signal at red wavelength region (635 nm and 705 nm), where the cell autofluorescence (at 705 nm) is lower. The intensity of this fluorescence, with the highest peak at about 635 nm, is related to the concentration of the photosensitizer within the cells. OAT cells distinguished by fluorescence peak at about 673 nm (red arrow, fig 3.10), and that is mostly related to

photoprotoporphyrin [14]. Fig 3.11 shows a 2.5-times higher PpIX fluorescence intensity in SW1710 tumor cells as well as in 293 cell. Analyzing the distribution of PpIX fluorescence intensity ratios between tumor and normal cells (HEK293) at 635 nm (representing PpIX accumulation). Fluorescence intensity is on average 8-15 times greater in malignant lesions than in healthy cells [70].

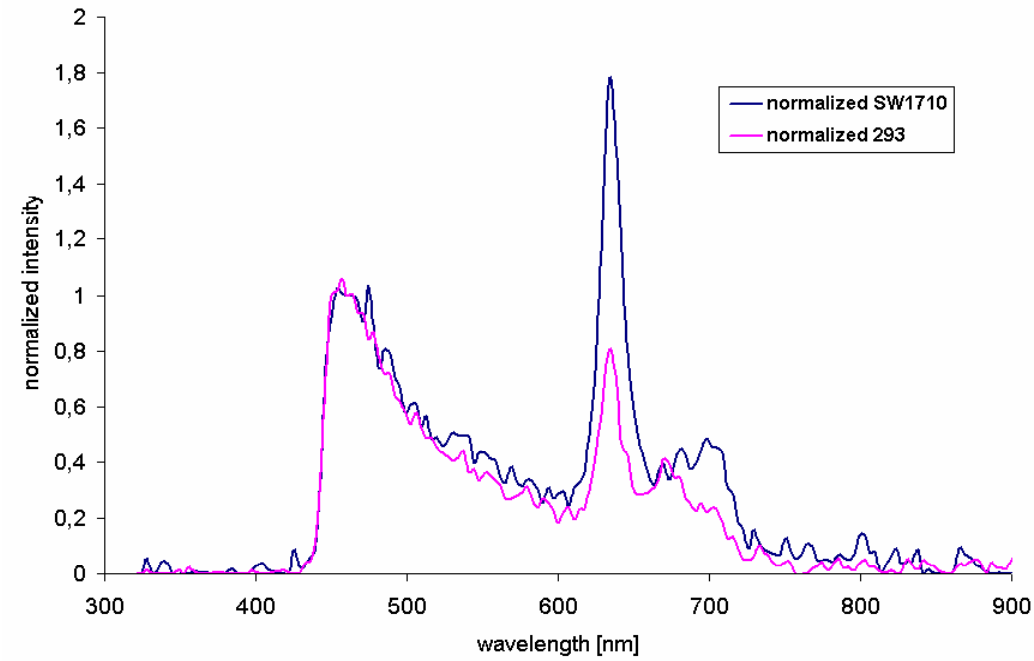


Fig 3.11 Fluorescence intensity (normalized to 460 nm peak) of the PpIX accumulation in 1E3 SW1710 and 293 cells/ 20µl (in haematocrite capillary), after incubation in the darkness at 37 °C with 2 mM 5-ALA for 3 hours.

The amount of pellet in the sealed haematocrite capillaries, indicted to concentration of cells in 20 µl is shown in fig 3.12 a, b.

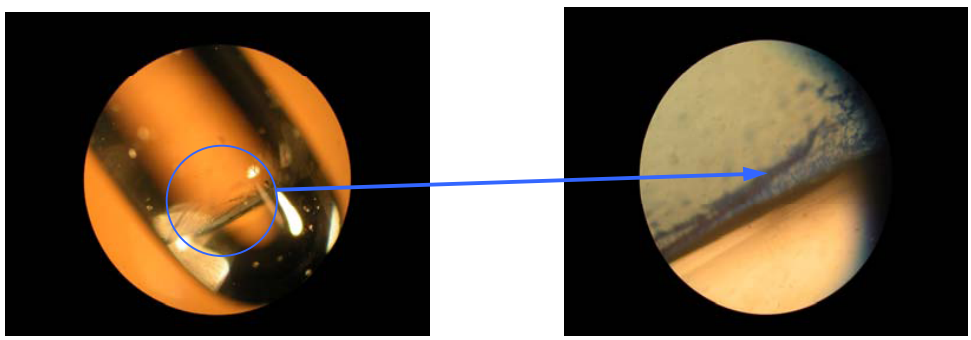


Fig 3.12 (a) sealed haematocrite tube, 10x enlarged (Light microscope, Nikon E200), methylene blue stained cell pellet of 200 OAT cells (10E3/ml as final concentration) after 3 minutes centrifugation in microhaematocrit centrifuge HAWKSLEY instruments. Blue arrow identifies the cell layers under high power.

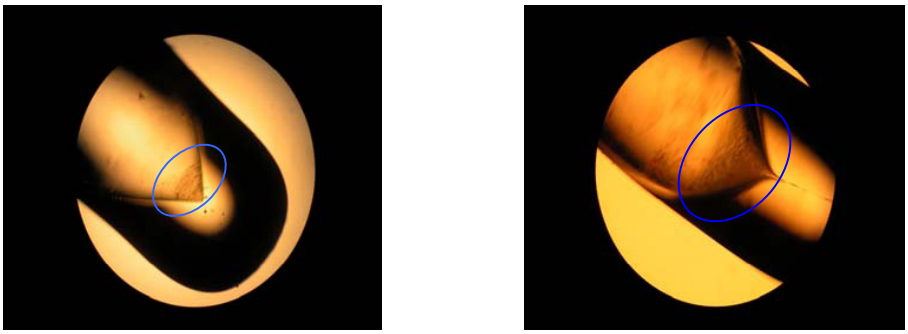


Fig 3.12 (b) Cell pellet of about 500 SW1710 cells (30E3/ml as final concentration) after 3 minutes centrifugation (left). (right) 10E3 SW1710 cells (0.5E6 as final concentration).

3.5 Saliva and exfoliative oral epithelial cells autofluorescence

3.5.1 Saliva samples

From 37 volunteers with different ages (18-45 years old) and oral hygiene. We have collected 30 sample of saliva and 50 brush biopsies from healthy volunteers. The investigation showed, cellular PpIX accumulation in the saliva and oral epithelial cells, that are isolated by brush. The PpIX can be an efficient photosensitizer. Maximum fluorescence intensity of the saliva and oral cells has been detected at 635 nm. Theoretically, the peak has been not detected in the nucleated cells without 5-ALA. About 200 species of bacteria live in the oral cavity, as a normal flora that, attached to (colonized) the epithelial and nonepithelial surfaces, or swim in the saliva. The result of saliva analysis showed different fluorescence and concentration of bacteria (table 3.3).

Table 3.3 Peak fluorescence intensities at the 405-nm excitation wavelength of ten samples of saliva from ten healthy volunteers after three hours incubation with 5-ALA.

Volunteer No.	Intensity of 5-ALA sample	No. of Epithelial Cells & Bacteria	Comments
1	1700 a.u	++	17 leukocyte
2	1500 a.u	++	
3	6400 a.u	+++	Root and dentine carious.
4	4300 a.u	+++	gingivitis
5	1760 a.u	++	Large number of bacteria still live after measurement by spectrometer
6	1250 a.u	++	Pregnant
7	1750 a.u	+++	
8	3450 a.u	++++	Large number of epithelial cells (large amount of pellet)
9	900 a.u	++	
10	700 a.u	+++	considerable amount of pellet

500 µl of Saliva with 500 µl DMEM without indicator were added. The 1ml sample incubated in the darkness at 37°C for 3 hours in water bath incubator. The measurements started by optical probe, after centrifugation (1000 rpm for 3 minutes). In spite of absence of 5-ALA, all samples from all volunteers, except samples of 3 volunteers revealed porphyrins (PpIX) peak after incubation. The pellet of saliva sample is stained by Acridinorange / Ethidiumbromide to quantify (on fluorescence microscope) the different concentration of strains of bacteria, further more, the number of epithelial cells. Moreover, this variation exhibited different fluorescent intensities peaks at 635 nm. In the evaluation of the 30 samples, 12 samples showed high fluorescence in the red region. The microscopic analysis observed high number of bacteria. A correlation has been found between, the high fluorescence and the presence of streptococcus salivarius, actinomyces and lactobacilli. These kinds of bacteria, related to dentin and root carious [20], [6]. And that was the oral situation of clinical diagnosis of oral cavity of 12 volunteers. Fig 3.13, shows the different fluorescence intensities of saliva sample from 35 years old male, with root carious and gingivitis.

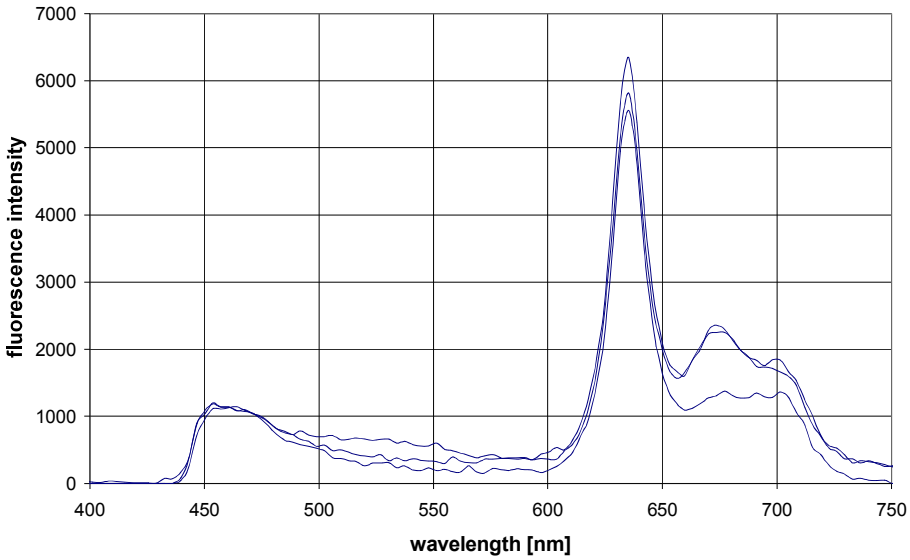


Fig 3.13 The different fluorescence intensities of saliva sample was excited at 405 nm and fluorescence emission was scanned from 550 to 750 nm. Maximum fluorescence intensity has detected at 635 nm, as well as the 5-ALA treated cell samples.

The supernatant was microscopically analyzed as well. The fluorescent image (fig 3.14), from the same volunteer, clearly illustrated the high number of actinomyces Israeli, S.mutans and lactobacilli bacteria, and this increasing; related to dentin and root carious. Moreover, the presence of small number of squamous epithelial cells that constantly is shed off oral mucosa every 24 hours, in addition to nutrient debris, enzymes and the electrolytes that are found in the oral cavity. Eight volunteers showed lower fluorescence intensity (PpIX peak) fig 3.15, and the pellet contains a considerable number of oral epithelial cells.

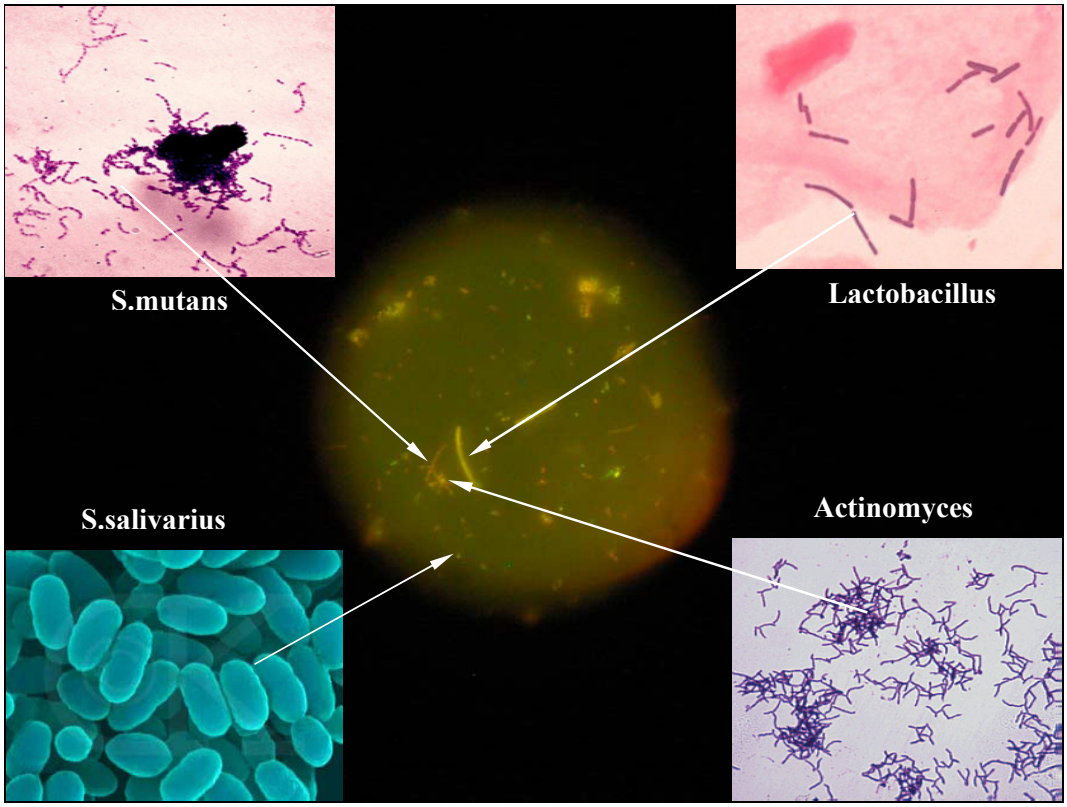


Fig 3.14 Supernatant saliva sample, fluorescent image of different bacterial species that are related to dentine and root carious [20].

Moreover, streptococcus salivarius represents most of bacteria in the eight samples. Seven samples from seven volunteers revealed difference intensities according to the situation of oral hygiene level. The saliva sample from irregular tooth brush user, showed high fluorescence due to increasing of number of streptococcus sanguis and streptococcus mutans; both are considered to be responsible for dental plaque. Three saliva samples did not show any emission after incubation time. Clinically the three volunteers have good oral hygiene, with regular using of mouth rinse.

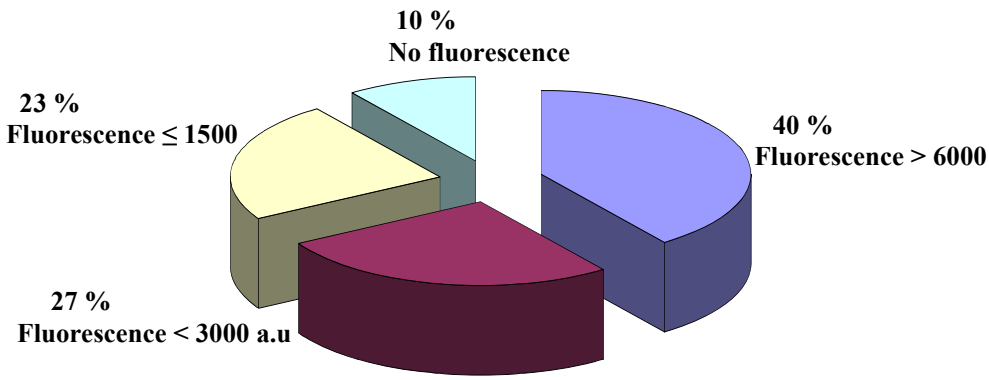


Fig 3.15 The ratio of PpIX fluorescence intensity in saliva samples.

3.5.2 Exfoliative oral epithelial cells

Oral epithelial cells were collected from 37 healthy adults, fifty samples have been prepared, followed by analysis by spectrofluorometr and microscope to quantify the epithelial cells. Oral microorganisms as well, that were adherent to epithelial cells or that have been collected from brush by centrifugation and analized on the fluorescece microscope. Three sampls (1ml/sample) were prepared from every biopsy and incubated in water bath incubator in darkness at 37°C. One sample served as a control and two were incubated with 2mM 5-ALA. The growth medium without indicator (from Sigma) is used. The medium showed very weak fluorescence, and did not interfere with the fluorescence assessment. A visual assessment of the control was the bacterial colony fluorescence, showing that oral flora emitted red fluorescence [71]. However, the analysis of the red fluorescence of 5-ALA samples showed very high fluorescence, as a result of excessive PpIX production that is excreted from bacteria into medium [72]. In fig 3.16 (a) oral epithelial cells sample without 5-ALA, in comparsion with tumor cell that served as a control. Both of samples were incubated for 3 hours at 37°C.

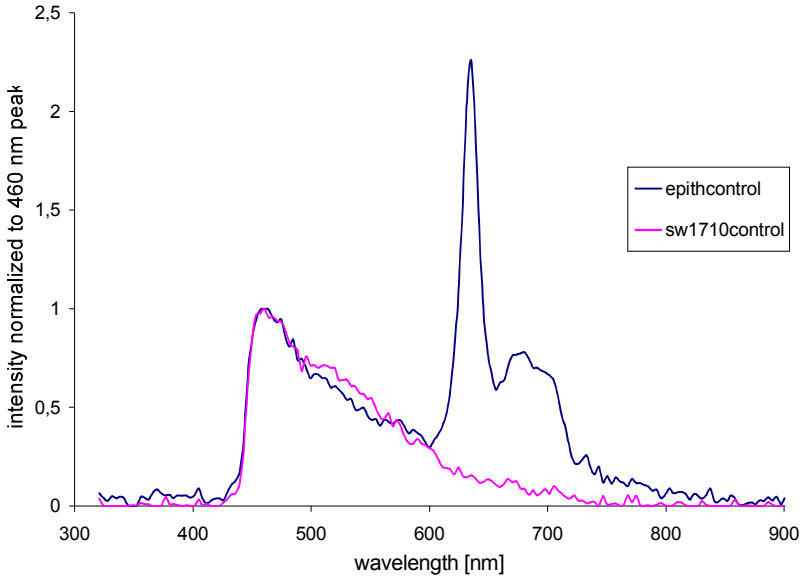


Fig 3.16 (a) Normalized fluorescence spectra for SW1710 cell line, and oral epithelial cells with bacteria related PpIX as a control, after 2 hours incubation at 37°C.

The centrifuged epithelial cells (collected from cheek) samples followed by pellet staining by Acridinorange / Ethidiumbromide. The microscopic fluorescence analysis showed the colonies of bacteria that strongly adherent to epithelial cells, moreover the colonies like streptococcus mutans and sanguis which need non epithelial surface (teeth) in order to colonize fig 3.16 (b). The adherence of various streptococci to different oral epithelial cells exhibited different autofluorescence intensities.

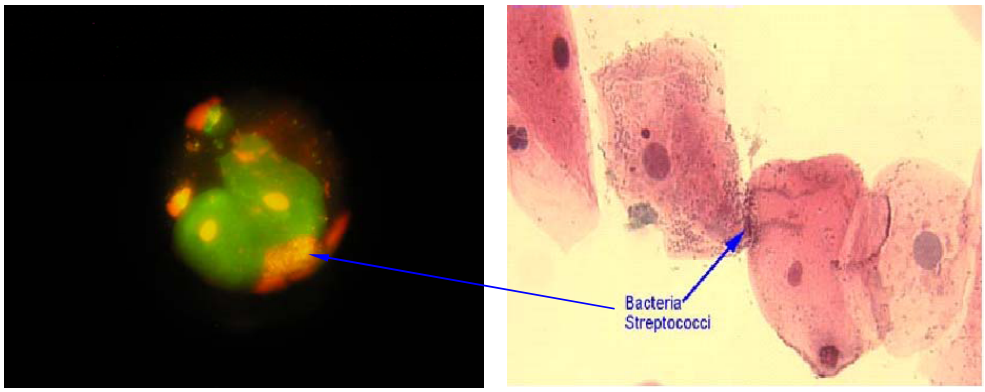


Fig 3.16 (b) Oral epithelial cells isolated by brush from cheek of healthy volunteer (right), bacteria (Streptococci) adherent to the surface of epithelial cell (blue arrow).

Direct production of endogenous porphyrins can be achieved by the induction of 2mM 5-ALA. The induction was, always performed by incubation of epithelial cells and the adherent bacterial species with 5-ALA in the dark at 37°C. Very high amount of porphyrins production are demonstrated by the height of fluorescence emission peaks that appeared in the 5-ALA treated epithelial cell samples and attached bacteria fig 3.17.

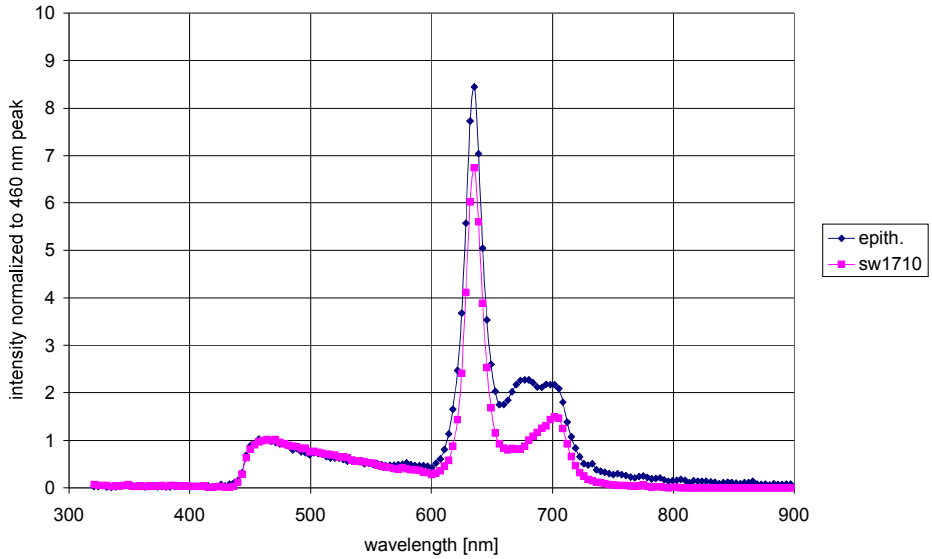


Fig 3.17 Normalized fluorescence spectra to 460 nm for normal oral epithelial cell (dorsal side of tongue) samples, and malignanat cell line SW1710 (at 405 nm excitation, after 3 hours incubation with 5-ALA).

All fluorescence measurements from the dorsal side of the tongue showed high signals, because of the presence of S.salivarius in high proportion (over 50 % of the streptococci), in addition to presence of S.sanguis [73]. Differences established between healthy locations. These differences represented relativity to species of bacteria that are adherent to epithelial cells. The epithelial cells were isolated from cheek, exhibited less fluorescence fig 3.18.

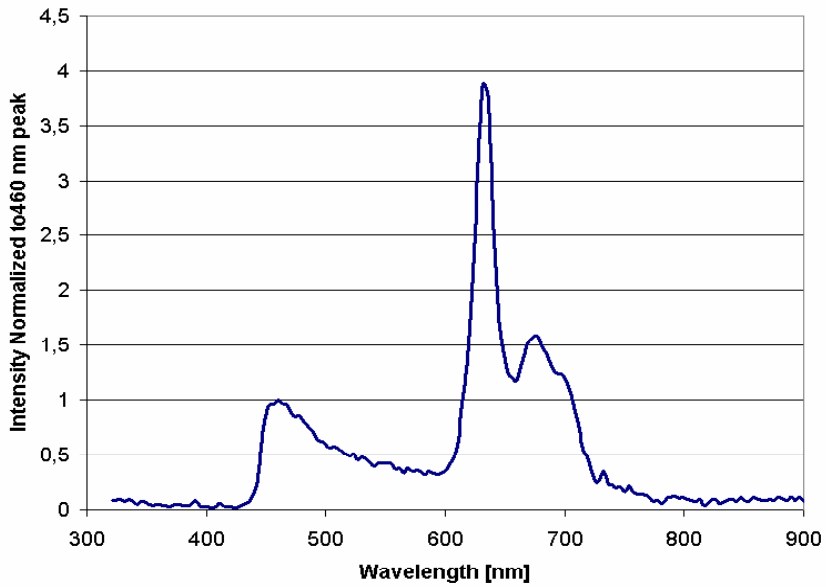


Fig 3.18 Normalized fluorescence spectra to 460 nm for normal oral epithelial cell, isolated from cheek. (at 405 nm excitation, after 3 hours incubation with 5-ALA).

The fluorescence intensity of brush biopsy sample indicated that the number of bacteria naturally attached to epithelial cells, varied greatly from site to site within the mouth [73]. Cheek cells generally averaged around 20 bacteria per cell, whereas cells obtained from tongue brush biopsy, usually averaged 100 or more per cell fig 3.19.

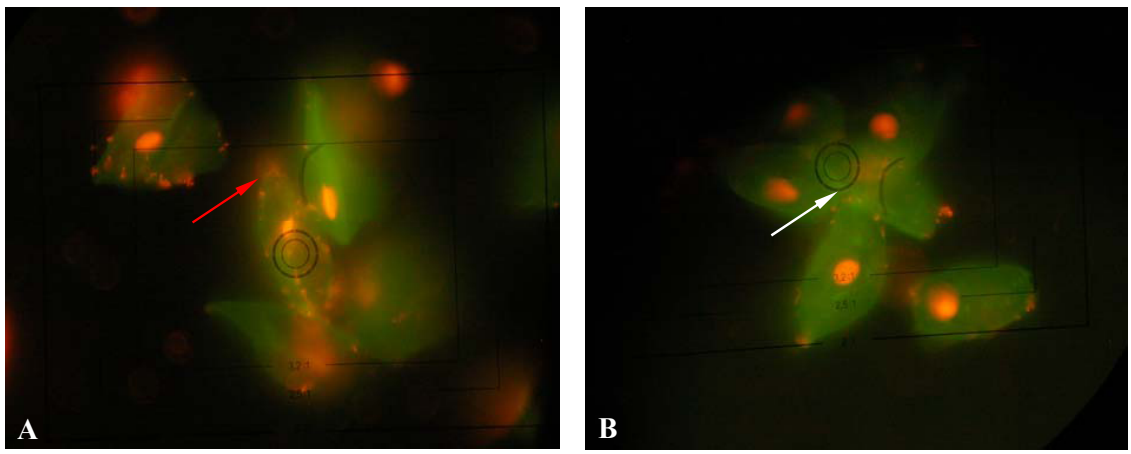


Fig 3.19 Brush biopsy of high oral hygiene female 30 years. (A) Large number of bacteria (S. salivarius) that is adherent to the oral epithelial cells of dorsum of healthy tongue (red arrow). (B) Less number of bacteria (S. mutans) isolated from cheek (white arrow).

Due to the presence of bacteria and their high ability to produce PpIX, the specificity of 5-ALA as method for fluorescent diagnosis is restricted [74]. Moreover, this may lead to false-positive findings. PpIX fluorescence in all samples (With(out) application of 2 mM 5-ALA) are investigated by (LIGA-Microspectrometer System). the different bacterial strains

that are found naturally in the oral cavity, considered as the main source of fluorescence. These results clarify that the suppression of bacteria fluorescence is necessary before starting the fluorescence measurements (as a method of diagnosis).

3.5.3 Suppression of bacteria fluorescence with PBS

Reduced fluorescence in brush biopsies (containing only healthy cells) are performed by incubation of 2mM 5-ALA treated samples in darkness at 37°C, for 3 hours. In addition to free 5-ALA samples served as controls. At the end of incubation, 1 ml samples are centrifuged and washed twice with PBS at pH 7.35. The measurements are carried out by excitation of the samples before and after washing. All the samples from all volunteers (except samples of three volunteers) produced porphyrins. Different intensities in different samples at peak 635 nm. And different concentration of microorganisms in the sample, and the number of epithelial cells after washing. The oral bacteria have different levels of adherence to oral epithelial cells. After cell pellet were washed three times in PBS, the result by spectrofluorometr was as following:

17 Samples Peak gradual decreased after washing

4 Samples Peak increased after washing.

11 Samples Peak did not change after washing.

5 Samples Peak disappeared after washing.

The procedure are repeated in triplicate, on different anatomical sites. The results were convergent. Most of control samples have been produced endogenous porphyrins and exhibited fluorescent intensities peaks at 635 nm fig 3.20.

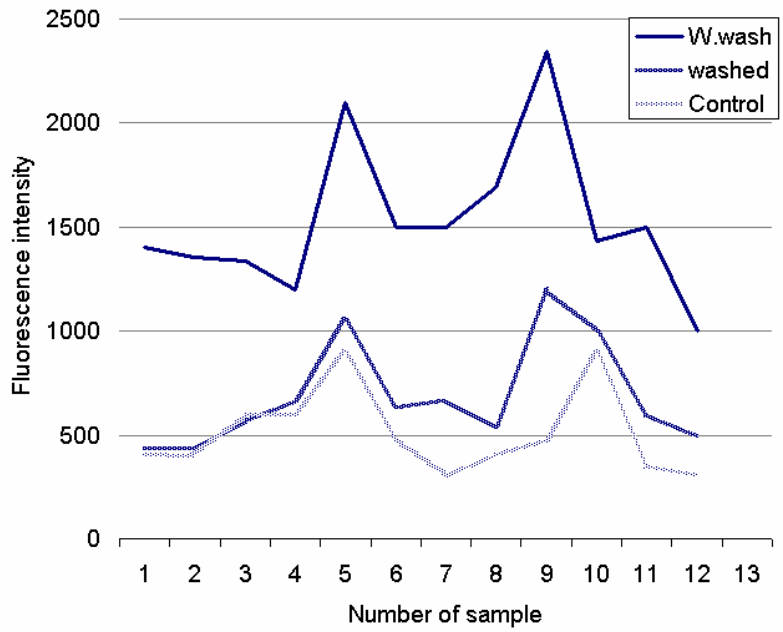


Fig 3.20. Fluorescent intensities of 5-ALA treated samples and control after three hours incubation time. Samples without washing (W.wash), three times washed samples in PBS at pH 7.35, and samples served as a control.

All samples have been stained by Acridinorange / Ethidiumbromide and were examined under fluorescence microscope to enumerate bacteria adherent to oral epithelial cells (fig 3.21), and quantified the amount of bacteria and cells. The oral epithelial cells have been counted (minimum 10^3 cells/ml counted) on inverted microscope (Ernst Leitz GmbH). The result under fluorescent microscope was the following:

17 Samples Minimum number of bacteria after washing in comparison to unwashed sample. Less adherent bacteria to epithelial cells.

4 Samples kind of bacteria (including streptococcus mutans, actinomyces Israeli, lactobacillus species), adherent to oral epithelial cells.

11 Samples The same adherent level of bacteria, before and after washing.

5 Samples Elimination of bacteria and oral epithelial cells by washing.

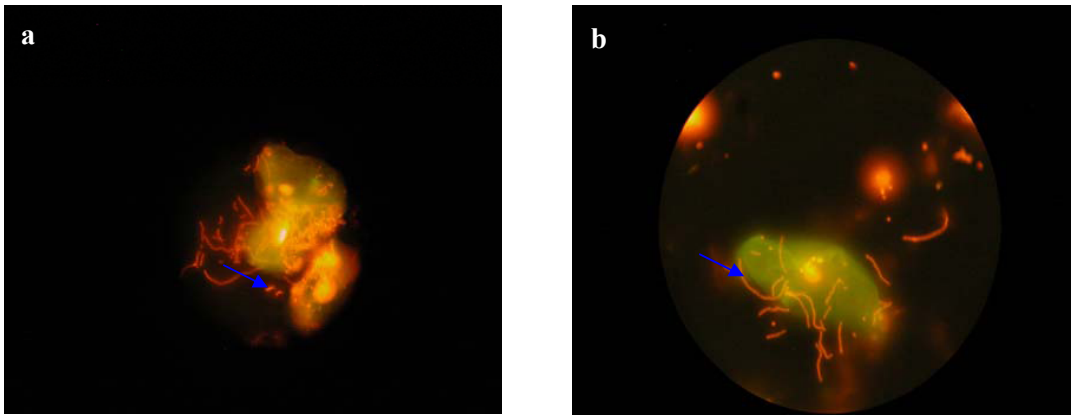


Fig 3.21 Streptococcus mutans and S. salivarius (blue arrow) adherent to the oral epithelial cell surface (isolated by brush from dorsum of healthy tongue). (a) Before washing in PBS, and (b) After three times washing in PBS.

The washed samples demonstrated changeful results. The decrease in fluorescence depends on the amount and species of bacteria. There was a moderate decrease in fluorescence of the 5-ALA treated washed sample as compared with unwashed samples. The results indicate more porphyrins synthesis in the 5-ALA incubated oral brush samples. Three hours incubation showed accumulation of porphyrins in the cell, and concomitant excretion of porphyrins from bacteria into extracellular medium [75]. The intracellular porphyrins (mostly coproporphyrin) [76], exhibit the fluorescence. The significant decrease depends on the bacterial species, moreover the adherent level to epithelial cell. Hence, the suppression of bacterial fluorescence with washing in PBS, might not become reliable as a method for bacterial suppression.

3.5.4 Suppression of bacteria fluorescence by chlorhexidine gluconate

Due to the ability of chlorhexidine to combat both gram positive and gram-negative bacteria, further more it is both bacteriostatic and bactericidal [76]. The selection of chlorhexidine gluconate (CHX) was more convenient. Before starting 5-ALA application, three groups of samples have been prepared. First group (1) of samples, the cells were isolated after instruction of volunteers to rinse the mouth by PBS. Second group (2) of samples, rinsed the mouth by chlorhexidine (D-gluconate) forte 0.2% (0.4 mM/l). Third group (3) the samples incubated with 10 µl of different concentration chlorhexidine forte (for 30 minutes before application of 5-ALA). We have found that the fluorescence spectrum from group (1) did not show any significant result. Whereas group (2) have shown significant fluorescence reduction in samples that is related to high oral hygiene (f ig 3.22).

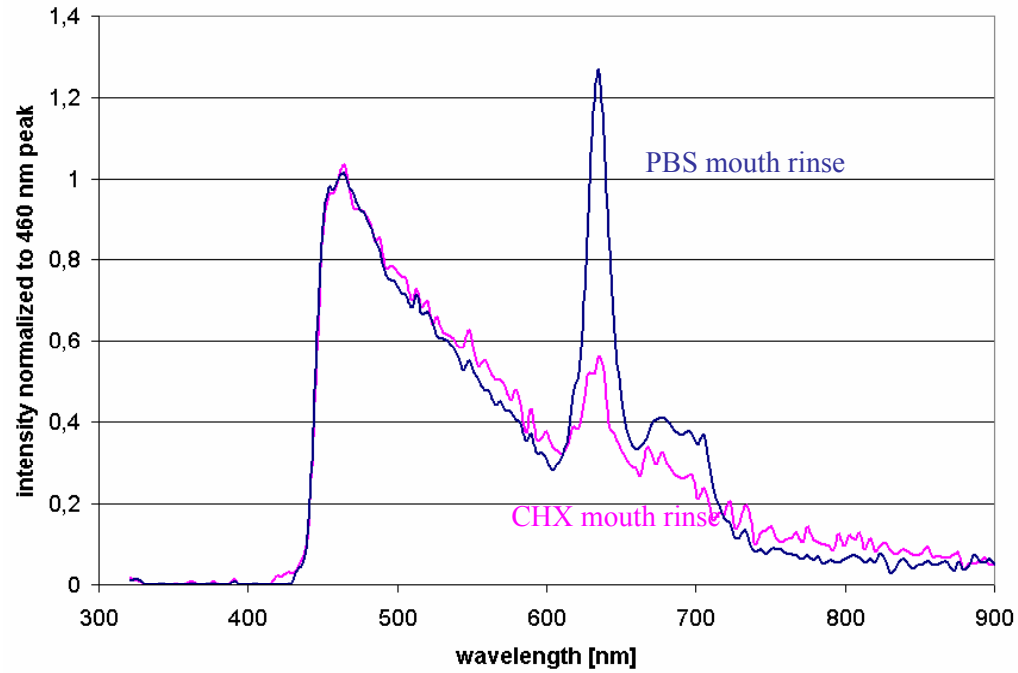


Fig 3.22 Normalized fluorescence intensity of epithelial cells after 2 hours incubation with 2 mM 5-ALA. The treated samples have preceded by (30 minutes) mouth rinses with PBS and chlorhexidine D-gluconate 0.2% (CHX). The volunteers, healthy male 37, female 25 years old. high oral hygiene was observed in the second volunteer.

The elmination of bacteria by PBS rinsing is restrictive to bacteria that are swim in saliva. A highly significant difference between the maximum PpIX fluorescence intensity in the second group The PpIX fluorescence differences is due to the different levels of oral hygiene fig 3.23. The colonies of bacteria did not show significant growth after incubation of samples that are isolated from the high oral hygiene volunteers. We have compared the samples before and after incubation for the group 2; in regardless of the species of bacteria, the number of microorganisms was smaller than that are observed in poor oral hygiene.

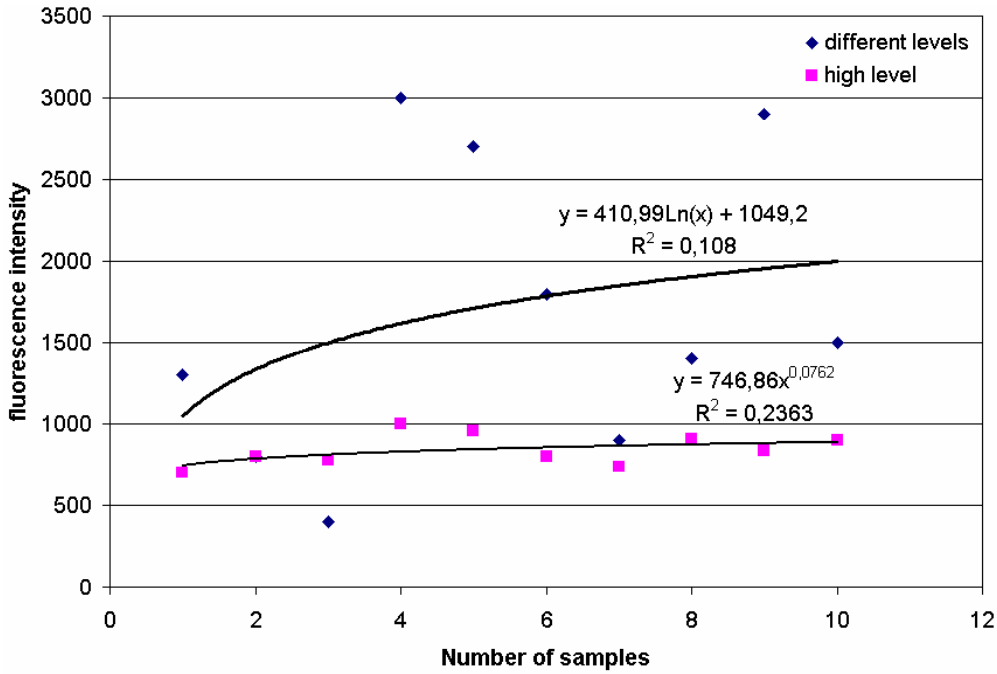


Fig 3.23 Fluorescsnce intensity of epithelial cells after 2 hours incubation of 5-AlA treated samples (the incubation is started 30 min after mouth rinse by chlorhexidine D-gluconate 0.2%). The ten samples are prepared from high oral hygiene volunteers. Other samples represented different oral hygiene (score 2, 3 OHI and score 2, 3 ,4 periodental index.)

Comparing both groups of 20 brushing samples: (10 high oral hygiene) and (10 different oral hygiene) the PpIX fluorescence intenstiy level in other ten samples was significantly higher. The samples in the third group, that are incubated for two hours with different concentration of chlorhexidine D-gluconate, showed different results. Several concentrations have been tested in brush biopsy samples; in order to determine the concentration of chlorhexidine for optimal differential labeling of epithelial cells and bacteria in the brush biopsies samples, while keeping the viability of epithelial cells. Three concentrations were tested in this study: 0.2% (0.4 mM), 0.1% (0.2 mM), and 0.05% (0.1 mM). The most suitable concentration for further experiments are chosen. The same procedure is used for this set of experiments as already described in material and methods (2.7.3.2). The chlorhixidine as well as growth media, has only very weak auto-fluorescence. In comparsion with the other antibiotics, that is tested during experments, the chlorhixidine did not interfere with the PpIX fluorescence. Tetracycline is broad-spectrum antibiotic has tested as a strong bacteriostatic affects fastly on normal bacterial flora. On the other hand it has very strong fluorescence, that interferes with the PpIX fluorescence and other photosensitizers (fig 3.24). We are started the incubation after application of 10 µl of different concentrations of chlorhixidine, 30 minutes before application of 2mM 5-ALA followed by two hours dark incubation in waterbath incubator.

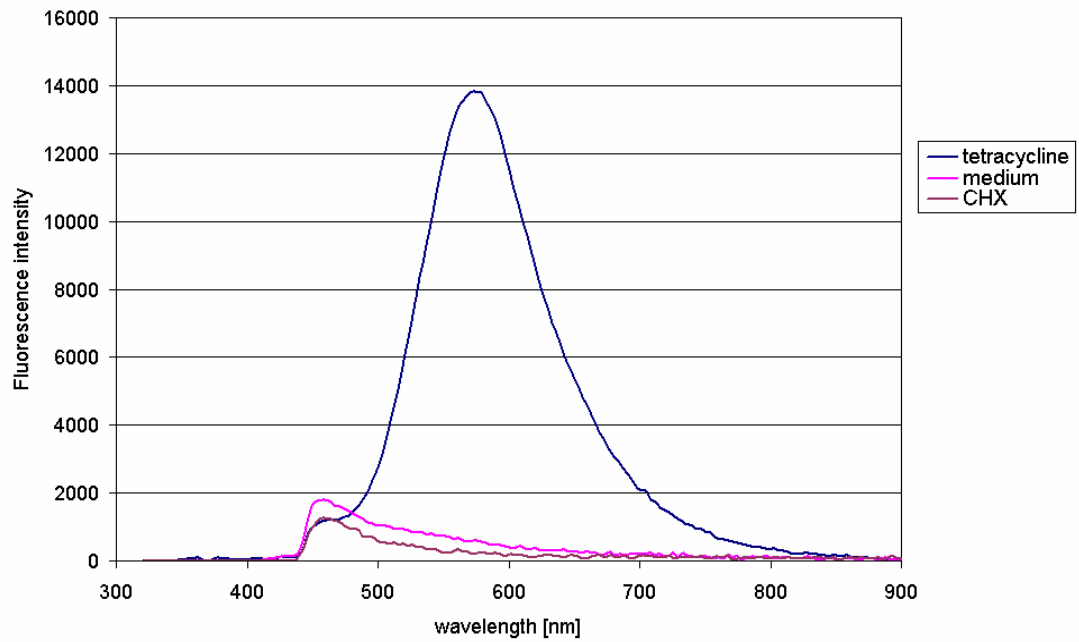


Fig 3.24 Fluorescence intensity of tetracycline, medium, and chlorhexidine gluconate.

All the samples that are incubated with CHX 0.4 mM are dropped fast to almost ground leveles of PpIX signal (fig 3.25). The bacteria related PpIX autofluorescence completely disappeared. Furthermore very low PpIX autofluorescence related to oral epithelial cells. After analysing of cell pellet on fluorescence microscope, the result translated the fast drop of PpIX signal. All the bacterial flora is dead beside most of epithelial cells fig 3. 26.

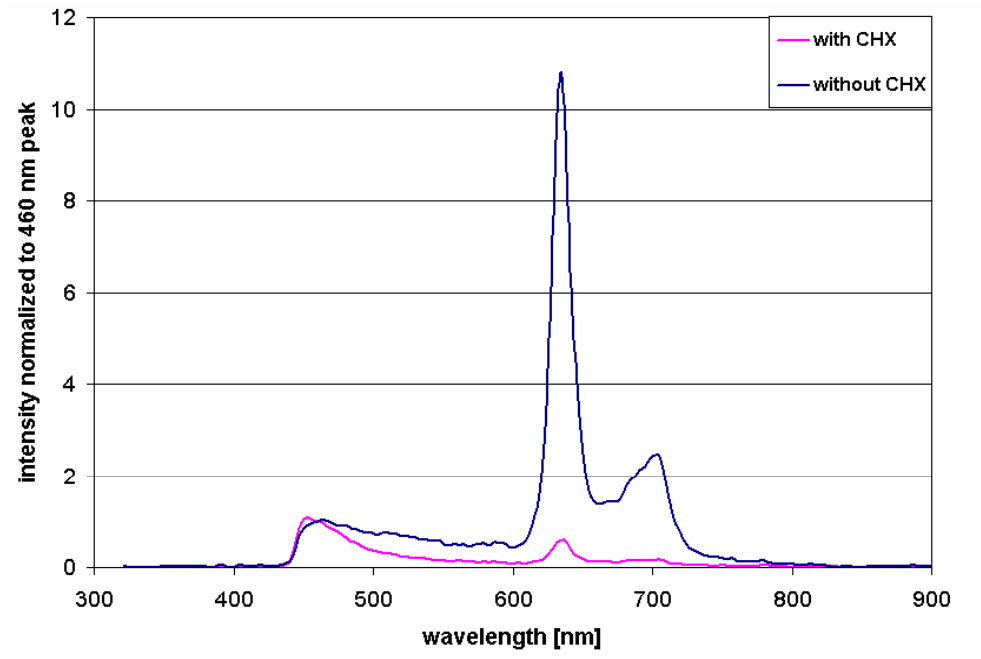


Fig 3. 25 High reduction of PpIX peak (magenta) after incubation of 5-ALA treated sample with 0.4 mM CHX (0.2%).

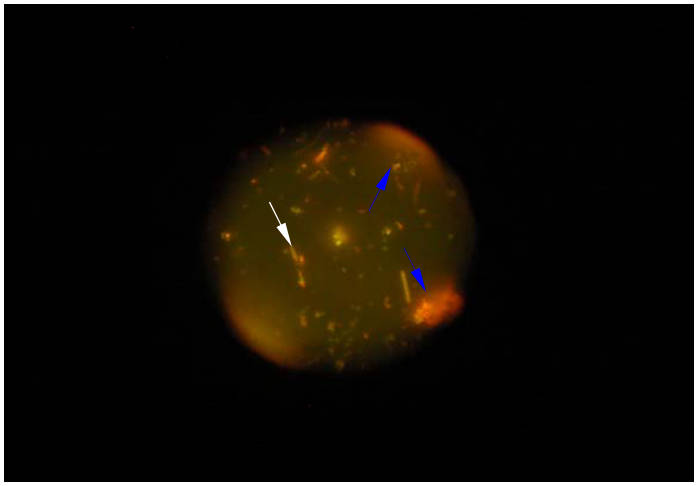


Fig 3.26 Dead bacteria (white) and epithelial cells (blue) in brush biopsy sample after 2 hours incubation with 2 mM ALA and chlorhexidine 0.4 mM.

The degree of cytotoxicity of chlorhexidine gluconate 0.4 mM on epitheial cells is determined as a function of the percentage of apoptotic cells [77]. We did not observe a significant difference in the malignant cells. We are recorded autofluorescence spectra from SW1710 and 5637 tumor cell lines, the same decrease of the PpIX peak is observed. PpIX fluorescence intensities in the samples incubated with 10µl chlorhexidine gluconate 0.2 mM (0.1%), are higher than samples of 0.4 mM CHX. as well as the vitality of epithelial cells was higher. The brush biopsies samples are incubated in 1ml medium containing different concentrations of chlorhexidine gluconate and 2mM 5-ALA. According to the vitality of SW1710 cells (fig 3.27), the highest data is observed among the chlorhexidine gluconate 0.1 mM (0.05%) at which optimal PpIX accumulation in epithelial cells can be measured.

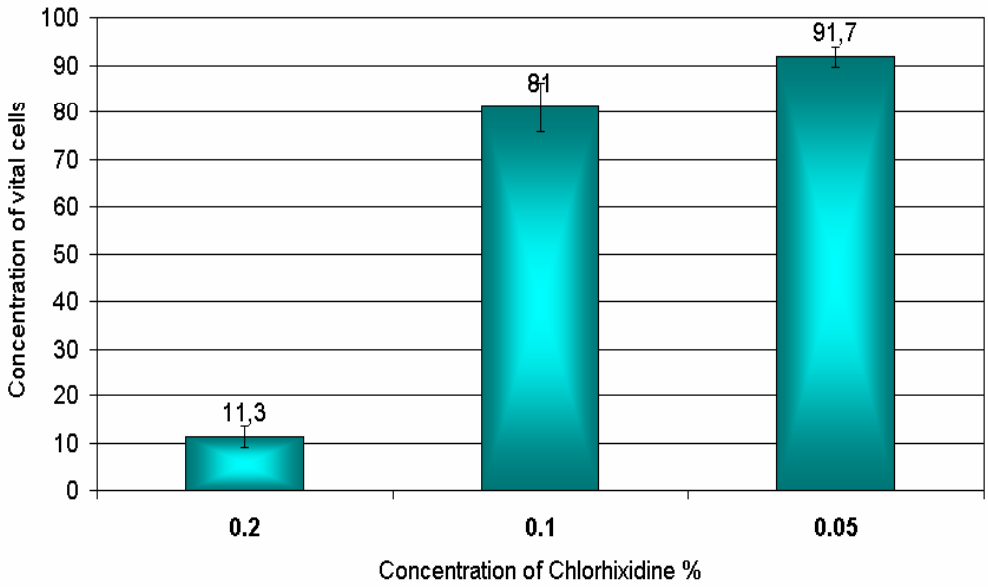


Fig 3.27 Concentration of vital cells (SW1710) after 2 houres incubatuion with different concentration of chlorhixidine.

Several brush biopsies from epithelial cells and malignanat cells samples, are tested in order to determine the concentration of chlorhexidine gluconate for optimal differentiation of tumor and normal cells while keeping the viability of the cells. The using of chlorhexidine 0.1% and 0.05 % in the tumor cell samples, the visualization of tumor cells and the evaluation of the pharmacokinetics of 5-ALA-induced PpIX fluorescence in chlorhexidine treated samples were greatly enhanced in comparesion to 0.2 % (Fig 3.28 A-D).

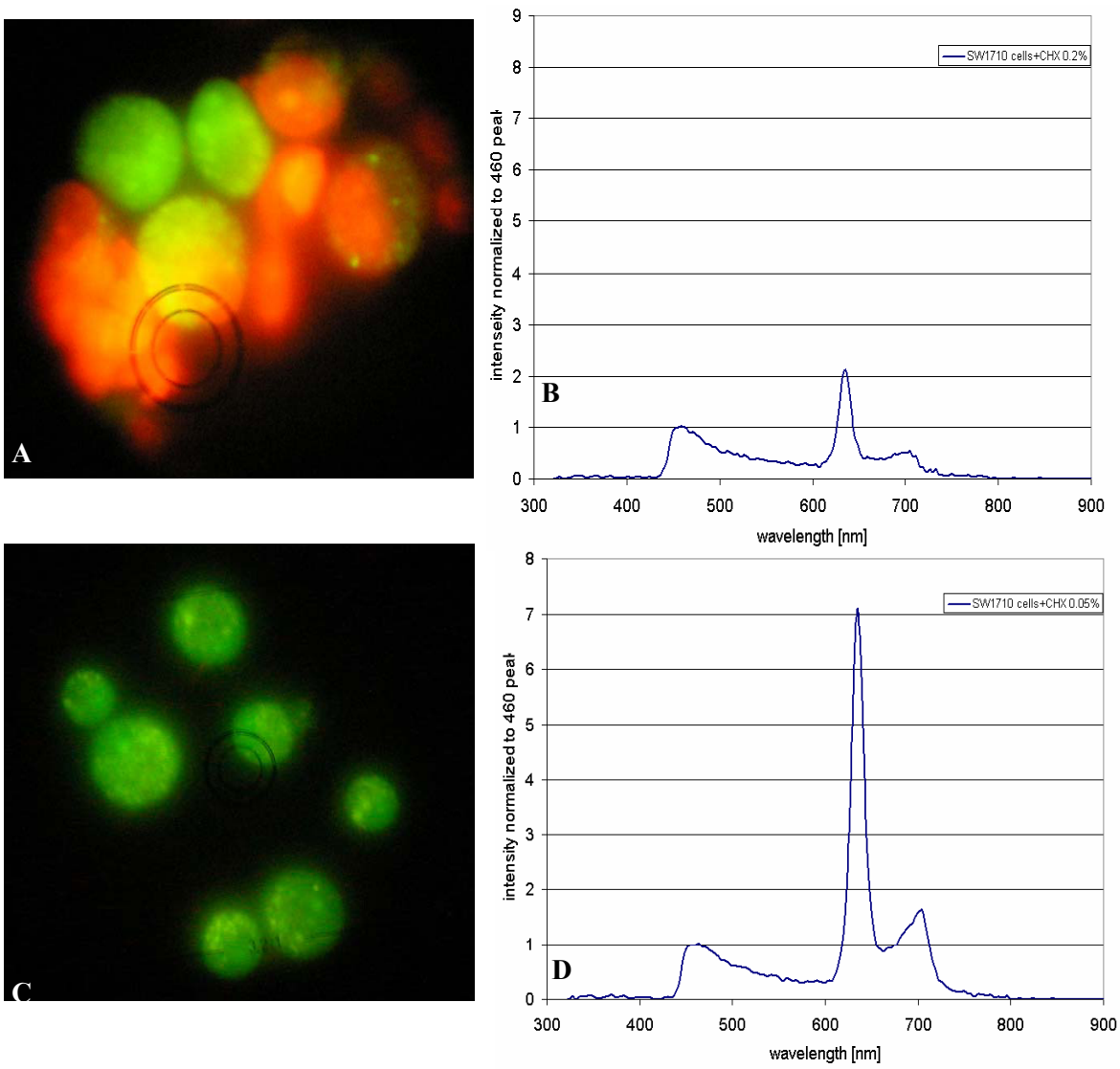


Fig 3. 28 Accumulation of 5-ALA-induced PpIX fluorescence in 0.5E6 / 1 ml SW1710 cell samples consisting of 0.4 mM (0.2%) and 0.1 mM (0.05%) chlorhexidine gluconate. The cell sample incubated with 2 mM 5-ALA for two hours. Pictures were taken under fluorescence microscope after staining with Acridinorange / Ethidiumbromide. Note the dead cells (red stained cells) (A) and PpIX fluorescence of the tumor cells sample (B), after incubation with 0.4 mM CHX. Vital healthy cells (green stain cells) treated with 0.1 mM CHX (C) and maximal PpIX fluorescence in the tumor cells (D).

No significant decrease of PpIX levels in tumor cells is detected between the different cell samples that have prepared with(out) 0.1mM chlorhexidine gluconate. However, a significant difference in the PpIX accumulation has been found in normal epithelial cells. The PpIX fluorescence in oral epithelial cells after incubation with 0.1 mM chlorhexidine gluconate are well differentiated (fig 3.29).

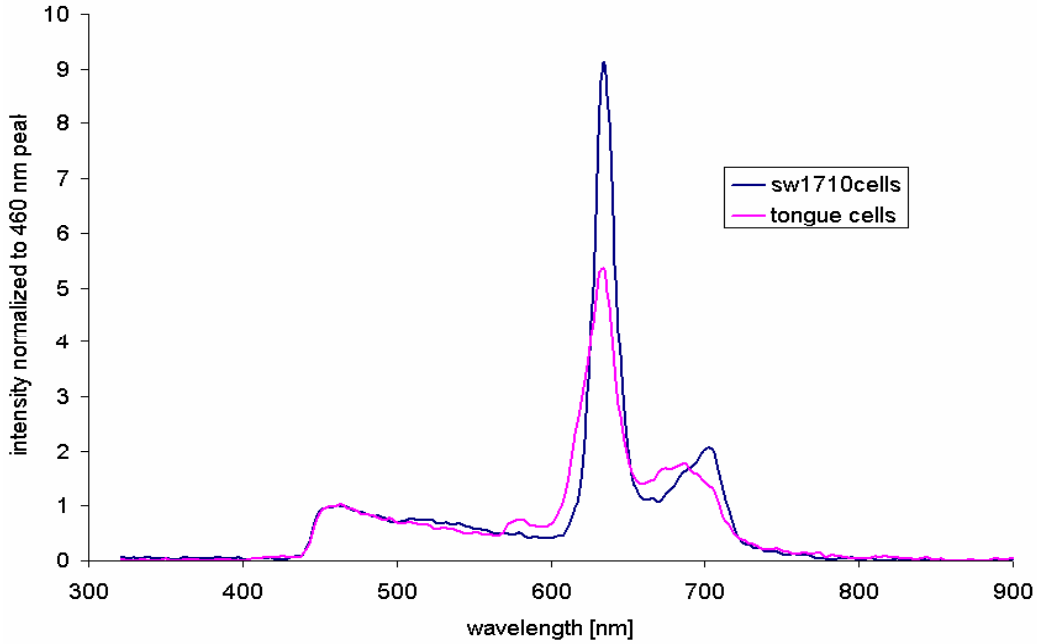


Fig 3. 29 Well differentiated autofluorescence spectra were normalized to 460 nm peak, for SW1710, brush biopsy cells of lateral border of tongue, after two hours incubation with 0.1mM chlorhexidine, followed by 2mM 5-ALA (after 30 min). Insignificant decreased of fluorescence signal of established cancer cell line (dark blue) and epithelial cells (Magenta curve).

Summarizing the results of the in vitro measurements of PpIX accumulation in brush biopsy samples and saliva samples as well; in the samples that are treated with 2mM 5-ALA and 0.1 mM chlorhexidine gluconate, it is founded that the maximal PpIX fluorescence intensity in cells samples did significantly differ between tumor and oral epithelial cell samples, and between oral epithelial samples as well (fig 3. 30). Maximal PpIX fluorescence intensities in tumor and in oral epithelial samples were served as a control samples and that are treated with 5-ALA only. Porphyrine like fluorescence is observed frequently, espicially in control samples. The chlorhexidine treated samples have been showen low level of PpIX fluorescence peak, that are produced by residual vital bacteria. The fluorescence images have showen non vital bacteria after two hours incubation time. Furthermore there is no influence on the vitality of epithelial cells (fig 3.31). The evaluation of the cytotoxic potential of 0.4mM chlorhexidine used in the experiments is of clinical relevance. Ideally, 0.1 mM of chlorhexidine is well tolerated by vital cells that, subjected to investigation in vitro.

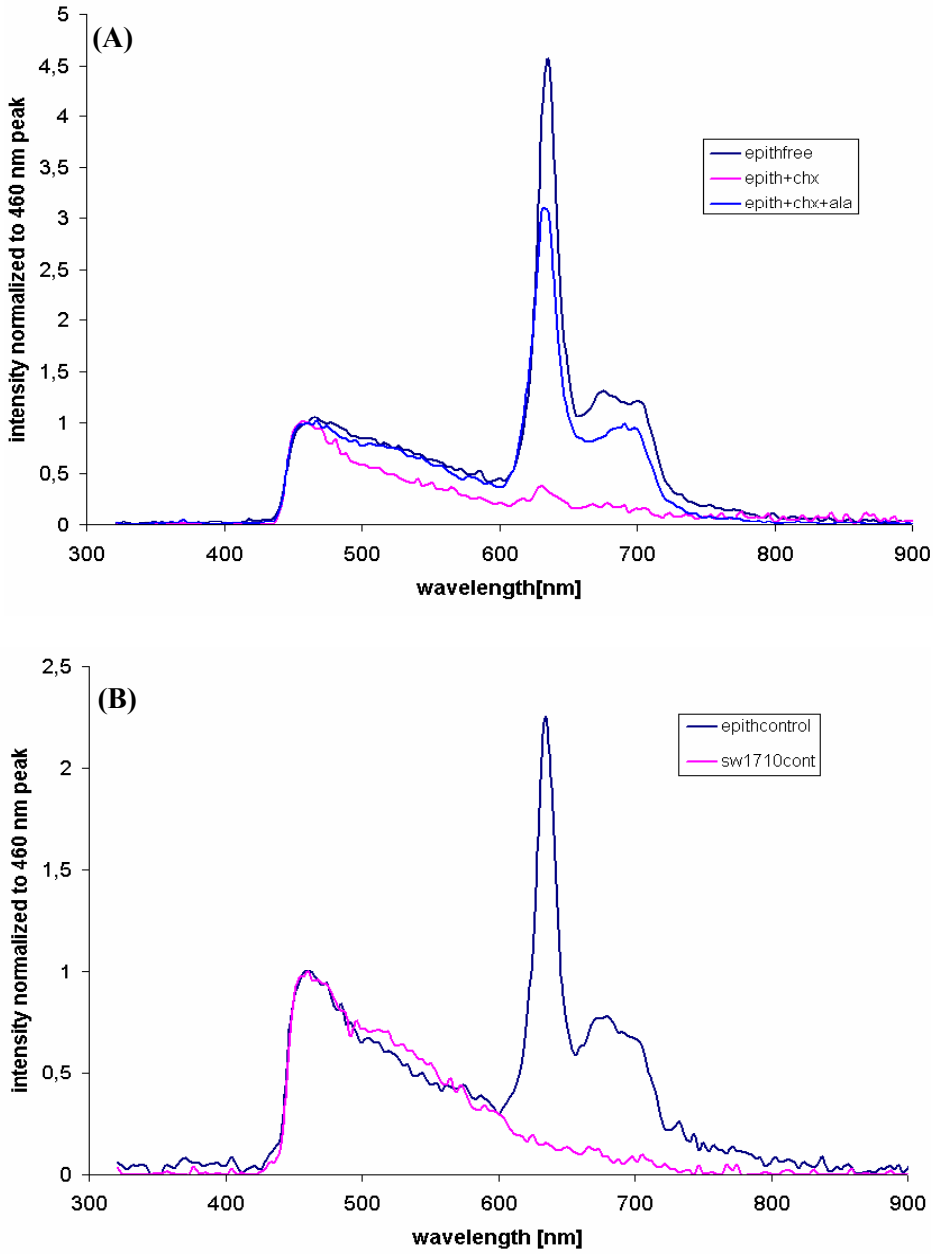


Fig. 3.30 Normalized fluorescence spectra to 460 nm of normal oral epithelial cell samples (at 405 nm excitation, after 2 hours incubation). (A) Strongly reduced fluorescence in brush biopsies (containing only healthy cells), that were treated with 0.1mM Chlorhexidine (Magenta curve) as well with 5-ALA (light blue). (b) Normalized fluorescence spectra of SW1710 cell line, and oral epithelial cells with bacteria related PpIX as a control, after 2 hours incubation at 37°C.

In a study that we performed ourselves, we have recorded autofluorescence spectra from healthy volunteers and 293 cells, furthermore the established malignant cell line (OAT and SW1710). All the cell lines were subjected to different experiments, washed after incubation, incubation with chlorhexidine gluconate and have been measured by spectrofluorometer after 5-ALA treatment.

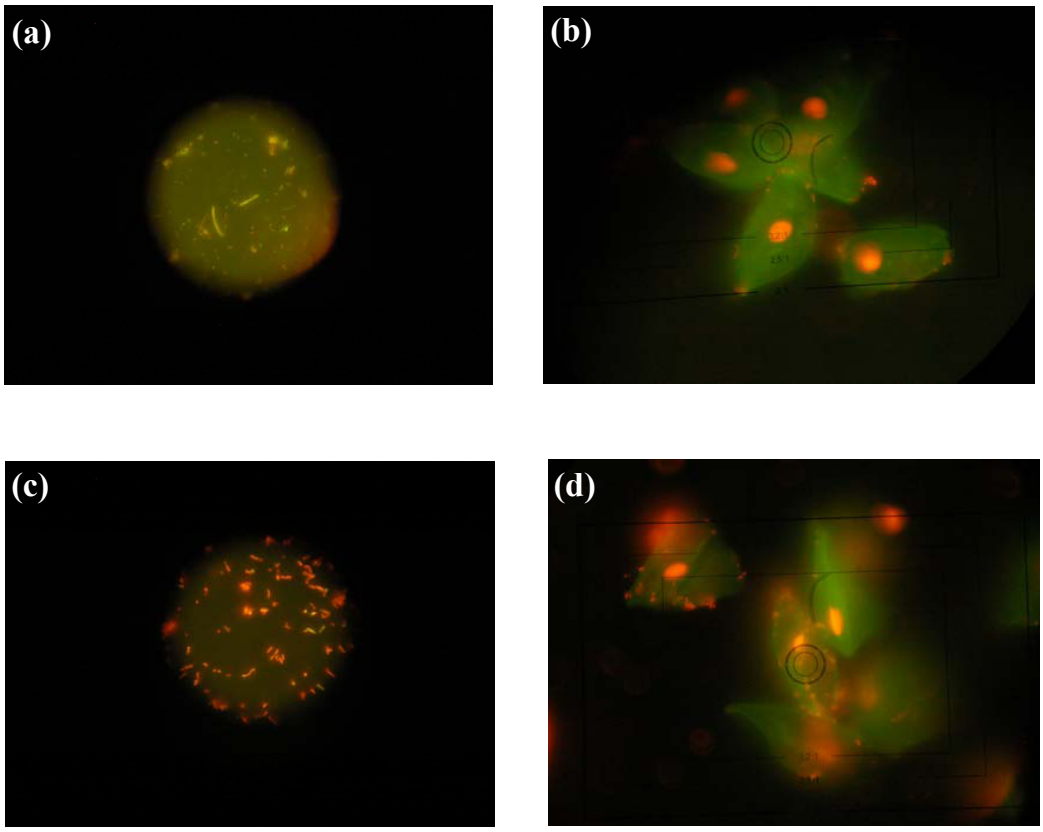


Fig 3. 31 Vital oral bacterial flora in saliva and brush biopsy samples incubated for 2 hours at 37°C (a), (b) indistinguishable green stained colonies of bacteria adherent to oral epithelial cells. Non vital bacterial flora in saliva sample (c). (d) Dead bacteria that is adherent to vital epithelial cell in brush biopsy sample, after 2 hours incubation with 0.1 mM chlorhexidine forte.

In comparison to cell washing as a method to reduce bacterial effect, the experiments were revealed that, by adding 1 mM chlorhexidine 30 min before incubation of brush biopsies with 5-ALA, increases reliability of the test by largely reducing the fluorescence signal due to the presecence of bacteria (fig 3. 32). According to data from our in vitro study, it is assumed that ability of bacteria to uptake and conversion of 5-ALA to PpIX requires a short time interval. Therefore, we proposed in this study a short incubation of chlorhexidine with time intervals from 30 minutes to 60 minutes for concentration 0.1 mM, followed by 120 minutes for concentration 2 mM 5-ALA. Mean fluorescence intensity of normal epithelium is (1484+263 c.u.) calibrated units (c.u.) [46], we could also observe this intensity limit in samples that were prepared from good oral hygiene that served as a control. Fig 3. 33 showed significant differences between chlorhexidine treated and untreated ten samples of healthy oral epithelial cells. One sample showed high intensity, because of chlorhexidine and 5-ALA treatment was took place at the same time (blue arrow).

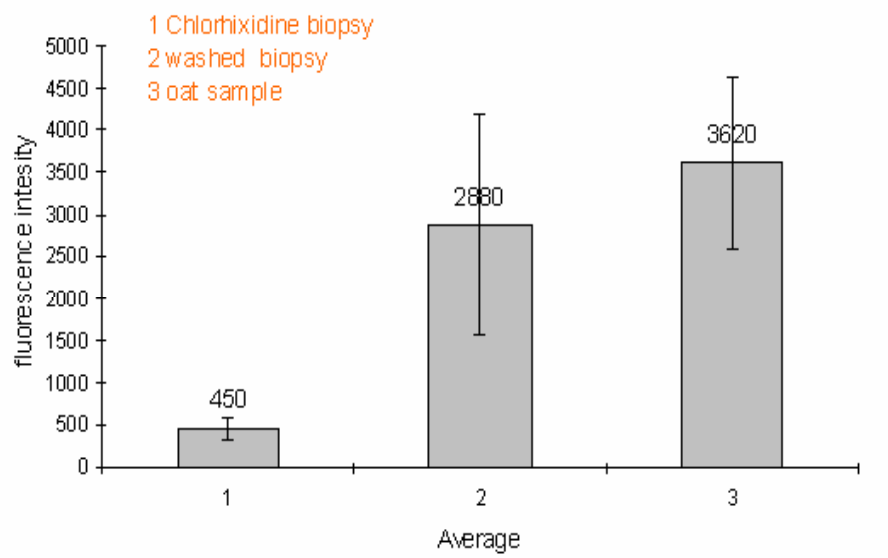


Fig 3. 32 The analysis of average of fluorescence intensities for 10 samples of oral epithelial cells (isolated by brush, incubated with 2mM 5-ALA and 0.1 mM chlorhexidine, PBS washed) and 500 OAT cells (5-ALA treated). Standard deviation is given for all samples.

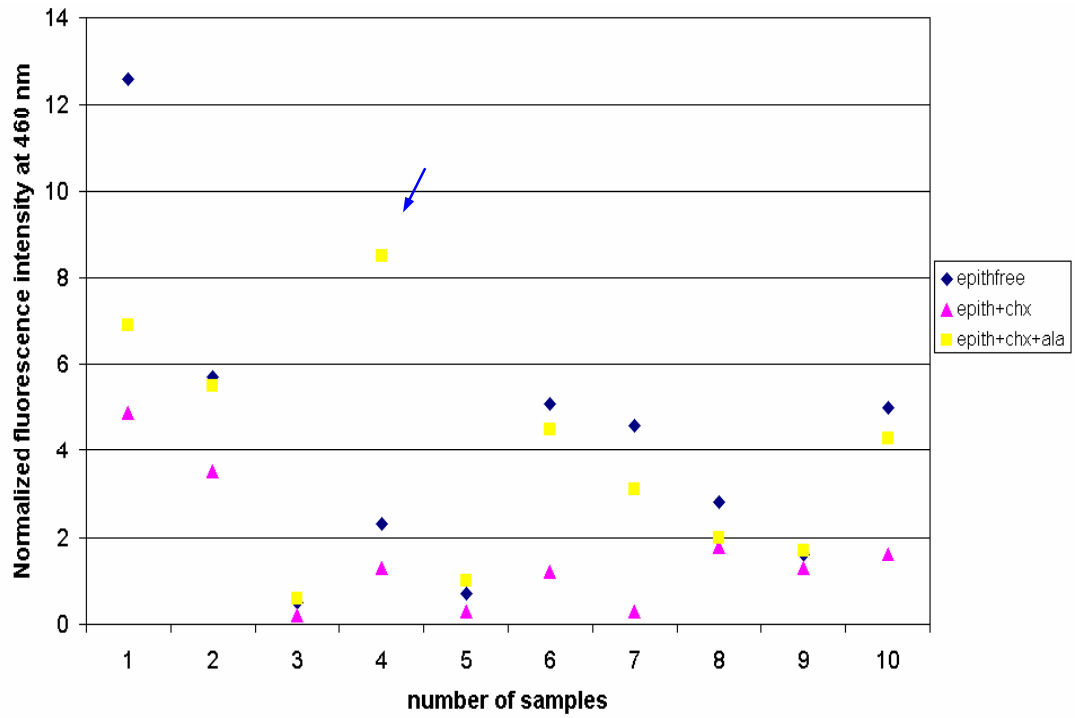


Fig 3. 33 Normalized fluorescence spectra for oral epithelial cell samples, strongly reduced of PpIX peak after 2 hours incubation with 0.1 mM chlorhexidine (magenta mark), as well with 5-ALA (yellow mark) in comparision to samples that served as a control (bacterial fluorescense), except one sample (blue arrow) that was incubated with 0.1 mM chlorhexidine and 2 mM 5-ALA at the same time.

4 Discussion

4.1 Fluorescence Detection of Cancer in Cell Suspension

The prognosis for patients with oral cancer significantly rises with an early detection of the malignant lesions, even for the experienced clinician, however, currently available diagnostic methods are fairly limited, as both inspection (macroscopic and endoscopic) and conventional imaging techniques some times lack to provide reliable results in the detection of tumors within the body. Therefore, new alternative diagnostic methods have been developed by using of dyes or fluorescent markers, respectively have been employed in order to achieve a better demarcation of the tumor boundaries. Still, none of these procedures has made its way into clinical routine and the medical profession generally hesitates in endorsing their use. Moreover, several other groups have endeavored to enhance the visualization of neoplastic changes within stratified squamous epithelium by means of autofluorescence photodetection [53]. Methods and results have been varying enormously and no general statements concerning the efficiency of this diagnostic procedure can be extracted. Alfano et al. [37] was the first to observe different spectral profiles of normal and cancerous tissues. Those alterations in both autofluorescence characteristics and intensities of neoplastic lesions and healthy mucosa can either be due to a variation in the concentrations of the fluorescent components or to changed morphological tissue structures. Thus, a thickening of the epithelium or overlying tumor tissue respectively may cause a decrease of the autofluorescence signals from the deeper layers of tissue. Ghadially et al. found a bright red fluorescence limited to the exterior of ulcerated tumors, which he identified to be a result of microbial porphyrin synthesis that can be wiped off the surface. He concluded that, in the presence of 5-ALA, many bacterial organisms that inhabit necrotic tissue are able to produce red fluorescent porphyrins. Even though researchers have extended their investigations of endogenous porphyrins in tumors to the oral cavity [52, 64, 71,], no routine diagnostic method on its basis has been developed so far. Our own results confirm, this studies and demonstrated the effects of microbil porphyrin on the photodiagnosis of oral lesions. 5-ALA has been investigated for fluorescence detection and localization of dysplasia and early stage malignant lesions in different body sites. An advantage of 5-ALA is that the spectral properties of PpIX are known, however, the administered ALA dose and the time interval between administration and the fluorescence measurements for a given route of delivery must still be optimized, because the tissue contrast depends strongly on these factors (Van der Veen et al., 1994; Heyerdahl et al., 1997; Loh et al., 1993). A great advantage of topical application compared

with systemic photosensitizers like photofrin is the rapid clearance of protoporphyrin and ALA from the skin. In addition, compared to systemic application of ALA, after its topical administration no significantly increased porphyrin levels in plasma were detected [46]. Systemic ALA administration might cause liver damage as well as neuropsychiatric disorders similar to symptoms of acute intermittent porphyria [79]. On account of this, there is a need of a tumor model in vitro suitable for pharmacokinetics studies. In the present work it has been shown the advantage of cell suspension to elucidate the pharmaco-kinetic properties of 5-ALA in order to optimize the drug delivery. The differences in the kinetics and PpIX fluorescence intensities in tumor and in normal cells have a clinical relevance. Our group demonstrated that 5-ALA penetrates across biological barriers of different malignant and normal cell lines in a significantly faster manner, generated a obviously higher formation of PpIX. Recently, a clinical investigation with 5-ALA has shown that 5-ALA induced fluorescence can be used for photodynamic diagnosis (PDD) in human bladder cancer. They applied 4 to 16 mM 5-ALA to assess the feasibility and the advantages of a topical ALA - based fluorescence photodetection in the human bladder. The results show that the application of 8 mM 5-ALA could provide sufficient PpIX fluorescence for reliable photodetection of malignant and pre-malignant lesions. Therefore, we applied 2 mM 5-ALA to 1 ml of cell suspension in order to reach the highest fluorescence intensity. We also investigated the time course of PpIX fluorescence and found for OAT, SW1710, and HEK293 cells an increased porphyrin fluorescence peak beyond 200 minutes. Up to thirty minutes nearly incubation no fluorescence could be detected. Then the fluorescence intensity rose and reached a peak between 120 to 180 minutes. After an application time of 240 minutes, the fluorescence intensity seemed to decline again. However, Our results are concordant to other investigations showing a peak fluorescence intensity after 3 to 4 hours depending on the cell concentration in 1 ml suspension [80, 70]. Based on these data we recommend an exposition-time of 180 minutes. The highest measured selectivity between 60 and 180 minutes in this fluorescence spectroscopy study corresponds to clinical data using fluorescence diagnosis and spectrofluorometer measurments of samples of small numbers (100-500) of established human tumor cells OAT 75, SW1710 (TCC) of the bladder and HEK293. The ratio of malignant cells versus normal cells is an important parameter for fluorescence diagnosis. Compared to our previous 5-ALA- induced porphyrin fluorescence study [67], we approved the efficiency of fluorimetric detection of a minimum number of malignant cells and normal epithelial cells. We observed a higher porphyrin fluorescence intensity within the 100, 200 malignant cell samples (2347 ± 381c.u.) as compared to HEK 293 cell line (1400 ± 330 c.u.).

This difference was statistically significant ($p<0,05$). 120 to 180 minutes after application porphyrin fluorescence in malignant and normal cell samples reached a maximal intensity. Tumor selectivity was highest in cell samples with average tumor to normal tissue ratio of 1:4 (fig 3.10). We performed a comparison analysis of more than 50 samples by microoptical probe that is used by our group in previous study, and the results demonstrated the ability of the microoptical probe to detect malignant cells in monolayer cells after application of 5-ALA [67]. with different concentration of cells that have been treated with and without 2mM 5-ALA, and 180 minutes incubation, followed by measurements with the microoptical probe that recorded the fluorescence intensity of three colour channels (red, green, blue). A higher PpIX fluorescence intensity at the red region was mostly achieved in the 5-ALA treated cells (OAT and SW1710) compared to the control samples. The ratio between red and green fluorescence rises after 180 minutes to level high enough to diffrentiate between the 5-ALA treated cells and control samples. In this study, 5-ALA was applied in cell suspension, other investigators have reported on systemic use of 5-ALA. However, it is desirable to avoid systemic 5-ALA application because of the possibility of dermal photosensitization in patients [81]. Topically and due to the limited penetration depth of protoporphyrin IX, it is, however, difficult to use the conventional fluorescence endoscopic system, to detect a tumor that is covered with tissue, except for the thin layer. Additionally, there is possibility of false negative results, particularly that related to cancer lesions were covered with normal appearing mucosa like the adenoid cystic cancer in oral palate [82]. An investigation by Svanberg et al., on head and neck cancer determined that the dosage of ALA must be kept low for maximal tumor demarcation. A higher dosage can cause excess biovailability in normal tissue. The fluorescence spectroscopy is a diagnostic modality with the potential to bridge between clinical examination and invasive biopsy. In the present study, spectroscopic data were obtained from 50 samples that were prepared from OAT, SW1710 cells and HEK293 cell line demonstrated the ability of fluorescence spectroscopy to differentiate between limited number of malignant and normal cells with high efficiency ex-vivo. Results of similar in vitro investigation by Dhingra et al showed the greatest differences between normal and abnormal tissue samples at 410-nm excitation. In our investigation, the 405-nm excitation wavelength produced the greatest number of red shifts (normalized peaks of malinant cell samples in the 635 nm region), which, when used in conjunction with the normalized peaks intensities of normal cell samples, provide an excellent signature for malignant cell samples.

4.2 Evaluation of Photodetection of Oral Cancer

4.2.1 Oral autofluorescence spectra In-vivo and Ex-vivo

Autofluorescence of tissues is produced by fluorophores that naturally occur in living cells after excitation with a suitable wavelength. The fluorophores can be located in the tissue matrix or in cells (e.g. collagen, elastin, keratin and NADH). The oral cavity is lined with a rich variety of mucosal types and the differences in structure between anatomical locations. These variations might translate into differences in autofluorescence spectral shape and intensity, consequently the oral cavity is unlike most other organs suitable for fluorescence detection of malignancies. For example, the cheeks, inner lip, soft palate, and floor of the mouth are lined with non-keratinized mucosa, while keratinized mucosa can be found on the hard palate, gingiva, and tongue. The presence of lingual papillae and taste buds makes the histological anatomy of the tongue unique. Furthermore, the palatal mucosa and gingiva are supported by bone, which might increase the reflection of incident and fluorescent light. Savage et al. examined five anatomical locations in the oral cavity and found statistically significant differences in emission wavelength intensity ratios [83]. Further more the influence of carcinogens like tobacco smoke and alcohol can cause long term damage of the oral mucosa ‘‘condemned mucosa’’ which can lead to ‘‘field cancerization’’ [84]. This process is reflected in the fluorescence characteristics of the upper aerodigestive tract. Other studies showed that spectroscopic changes do not occur only at the centre and border of lesions, but also in the surroundings, where no abnormalities are visible [85]. This suggests that the distinction between healthy and diseased tissue within a patient is not always well defined. Anatomical locations showed high fluorescence intensity in spite of the fact that these anatomical locations are spectroscopically comparable. This tells us that total fluorescence intensities are highly inconsistent, both intra- and inter-individually. There are several possible biological explanations for the large intra- and inter-subject variability in total fluorescence intensity. Fluorescence intensity can be influenced by intersubject variability in the amount of blood, with absorption leading to a wavelength-dependent decrease in fluorescence intensity. Some anatomical locations produced distinct autofluorescence spectra. For example, in 94% of all healthy volunteers the DST (dorsum of tongue) showed a fluorescence peak at 636 nm at 405 nm excitation. For relatively high 635 nm peak intensities, an accompanying smaller peak centred around 705 nm has observed. The 635 / 705 peak combination strongly resembled the in vivo emission of PpIX [66]. Our findings ex-vivo suggest that the variability in the presence of porphyrin-producing

microorganisms can influence the total fluorescence intensity by providing an additional amount of fluorescence at 635 nm. We frequently observed 635 nm peaks that we believe to be caused by porphyrins, which are fluorescent substances produced by living cells without application of photosensitizing agents. This porphyrin-like peak has been reported by other authors [53, 85, 86]. It seems generally accepted that it is caused by endogenous porphyrin production. This may be either by microorganisms that find a natural habitat in oral cavity like those in samples that are prepared from exfoliative biopsy of dorsum of healthy tongue (DST). The porphyrin-like peaks were found most frequently at this location (97% of volunteers). The presence of a porphyrin-like peak at this location probably explains the high fluorescence intensity rates in the DST in vivo and ex-vivo. We accord with many studies that likely assume the 635 nm peaks at the mandibular fold and the gingiva to be caused by porphyrins appearing in dental plaque [64, 71, 87]. The peaks appearing at the soft and hard palate in-vivo may be explained by the frequent contact of these locations with the tongue. This is plausible because at least part of the porphyrin like fluorescence producing substances is located on dorsal surface of the tongue. Another explanation for porphyrin-like peaks appearing at other locations than the tongue might be found in bacterial infections that are not noticed clinically [88]. The presence of porphyrin-like peaks in autofluorescence spectra of healthy oral mucosa cells, will lead to false positive classifications.

Our own examinations have shown a bright red fluorescence due to endogenous porphyrins on the cell pellet samples that collected from dorsum of tongue, gingival surface and buccal aspect of mouth, this observed “red fluorescence” represented bacterial porphyrin accumulations. we have investigated all the samples under fluorescence microscope after staining by vitality dye. The bacteria did not seem to be spread homogeneously over all mouth surfaces, the cells that were collected from dorsum surface of the tongue have showed more different colonies of microorganisms that heavily attached to the cells, in other hand the collected buccal cells were more clear and the amount of bacteria is less than other samples. We found that the variation in total fluorescence intensity is large between healthy volunteers for one specific anatomical site, and this was according to the level of oral hygiene (fig 3.22).

In this study we have been proved beyond any doubt that the porphyrin like fluorescence in oral epithelial tissue is due to different species of bacteria. The isolated cells that were collected by brush biopsy from different healthy locations in oral cavity, produced red fluorescent pellet with porphyrin like fluorescence. The phenomenon of red fluorescence is due to the presence of porphyrins, produced by the action of colonies of bacteria that are adherent to the epithelial cells [73]. From 37 healthy volunteers we have collected 50 brush

biopsies from healthy oral mucosa as well as 30 sample of saliva. We have observed cellular PpIX accumulation in the saliva. The fluorescence intensity of the saliva has been detected at 635 nm as cell samples. About 200 species of bacteria live in the oral cavity, as a normal flora that, attached to (colonized) the epithelial and nonepithelial surfaces, or swim in the saliva. The result of saliva analysis showed different fluorescence and concentration of bacteria (table 3.3). The saliva samples that have showed high fluorescence, related to the large number of streptococcus sanguis and streptococcus mutans, both are considered to be responsible for dental plaque, on the other hand the samples with weak fluorescence have been related to less number of bacteria, and this observed in good oral hygiene volunteers, with regular using of mouth rinse. According to some studies, the source of porphyrin in oral cancerous tissues is assumed to be not microbial contamination but cancer cell metabolite [86, 89]. These studies are based on some reasons to support their hypothesis from previous researchers [1 4, 9], the first reason: Porphyrins are assumed to be excreted by viable tumor tissues, accumulate, and become condensed in necrotic tissue, and seen so much more commonly in cancerous than in simple necrotic ulcers. Our investigation completely contradicts this hypothesis, the incubation of suspension samples of oral epithelial cells and hundreds of types of bacterial organisms, lead to accumulation of PpIX in suspension. This accumulation is due to the bacterial excretion of PpIX into media. The microenvironment of the oral cavity changes with oral or systemic disease that alter the number and proportion of flora [90], and these changes are responsible for revealed of porphyrin like fluorescence that has been observed during measurement by spectrofluorometer. The increasing the levels and activity of bacterial organism, in addition to oral microorganisms can include fungal, protozoal, and viral species that have been attached to oral lesions, lead to red fluorescent area in cancer lesion. We have to take in consideration the roughness of lesion surface that may play a role in accumulation of bacteria on the lesion surface (retentive area). Wherefore this porphyrin-like fluorescence was probably produced by microorganisms living on ulcerating or necrotic surfaces, which is consistent with the observation that the fluorescent materials could be wiped off. Other reason relay on the difference between the oral cancer and oral ulcer fluorescent phenomenon, that is much more commonly seen in cancerous than in simple necrotic ulcer [according to Sharvill´s study] and is assumed to be characteristic of oral cancer. If it is caused by microbial activity, a simple necrotic ulcer would also be expected to show the bimodal feature. This difference probably due to histological feature, the early oral carcinoma result from processes keratinisation and epithelial damage followed by rolled of border and ulceration, and at low power of epithelium is seen to invade deeply, as a result as,

more area was exposed to different bacterial colonies proliferation and the fluorescence intensity is higher. On other hand the ulcers typically affect only the non keratinised mucosa such as the buccal mucosa [13], sulcuses or lateral borders of the tongue, and the bacteria have less ability to adhere to these locations. Moreover the ulcers is destruction of the epithelium only and less colonies are attached to the surface of ulcers (i.e. away from lamina propria like the malignant ulcer). we plan to investigate oral epithelium samples from different anatomical locations (keratinised and non keratinised), they show significantly different autofluorescence intensities. However, the collected cells from the keratinised tissue like the dorsum of the tongue, was much larger than non keratinised tissue, like buccal mucosa. The fluorescent microscopic results showed that presence and increase of bacterial organisms is associated with porphyrin fluorescence (table 3.3). In other study was found only one third of the tumors examined (33.3%) to be mostly covered by strongly red fluorescing material. Yet, tumor discriminating abilities are fairly limited, as the observed "red spots" that represented bacterial porphyrin accumulations did not seem to be spread homogeneously over the lesion's surfaces [53]. The other reason: Lesions cured by chemotherapy or radiation therapy without any sign of recurrence did not show the bimodal feature. It is thought that the lesions must not contain cancer cells after treatments. Its well accepted that absence of bimodal feature is the result of suppression of microorganisms by chemotherapy and radiation, and both of them are maybe bactericidal (kill bacteria) or bacteriostatic (inhibit growth, thereby limiting numbers of infecting organisms to levels which the host defences can control) [91]. Thus, we do not advocate the autofluorescence for the detection of oral cancer. The term "autofluorescence" means here the fluorescence emitted by the tissue on irradiation with excitation light without fluorescent drug administration. Because oral tissues contain variety of mucosal types, these variations might translate into differences in autofluorescence spectral shape and intensity. However, autofluorescence spectra of healthy oral mucosa also highly vary in intensities for different and even within the same anatomical region of the oral cavity, which may highly affect the value of spectral data obtained. It cannot be truly satisfying for an accurate tumor detection alone, it should be additionally considered by the examining surgeon and should yield better results.

4.2.2 PpIX fluorescence after application of 5-ALA In-and Ex-vivo

For tumor marking in connection with fluorescence diagnostics, aminolevulinic acid (5-ALA) induced protoporphyrin IX has also been exploited. PpIX has a characteristic dual-peaked fluorescence emission in the red spectral region, with one high and narrow peak at about

635 nm and one smaller and wider peak at about 705 nm. 5-ALA based fluorescence diagnosis has been found to be promising for an early detection and demarcation of superficial oral squamous cell carcinomas. One important property of laser-induced fluorescence (LIF) using 405 excitation is that only a superficial tissue layer is probed, due to the small penetration depth of the excitation light. At this wavelength, the penetration depth is only a few hundred micrometers. The cancer lesions, however, might be a few millimetres thick. The information obtained by LIF in superficial layers of the lesion does not provide information on the status deeper down [65]. The use of exogenous fluorescence for tumour detection has been investigated for various organs. For the oral cavity, some promising results have been obtained. However, the use of exogenous 5-ALA has some major drawbacks [92]. A certain waiting time (incubation time) after application is necessary for the fluorophore to reach its optimal fluorescence intensity. Furthermore, the application of photosensitizers leaves the patient temporarily sensitive for light, which negatively affects his daily life. This makes the technique impractical, especially for use in regular screenings of high-risk patient groups. Finally, the specificity of the photosensitizers appeared to be less than expected [93].
5-ALA based fluorescence as a method of photodiagnosis has previously demonstrated high sensitivity, however this clinical trial showed a specificity of approximately 62 %. This specificity was mainly restricted by tumor detection in the oral cavity in the presence of bacteria. After topical 5-ALA application in the mouth of patients with suspicious lesions, red fluorescence areas were observed which did not correlate to confirm histological findings [74]. These results may lead to false-positive findings. In present study we examined the kinetics of the PpIX build-up, by spectrofluorometer and optical probe after administration of 2 mM 5-ALA given for diagnostic purposes. The build up in normal epithelial cells collected by brush from healthy oral mucosa were peaking after about 2-3 hours. In addition to malignant cells. When supplying 5-ALA, several bacteria can generate porphyrins and also be subject to subsequent photoinactivation. Excess porphyrin production is excreted to the medium [94]. The samples of oral epithelial cells collected from 37 healthy adults volunteers with different levels of oral hygiene. The primary aim of our fluorescence investigations has been to discriminate between cancerous and healthy cells. One could also have used the fluorescence data to, for instance, obtain information regarding PpIX concentration in the cells. In any case, the spectral fluorescence data recorded often have to be condensed to an indication of diseased or non-diseased cells. In doing this, both spectral shape and intensity should be considered in the analysis. Three hours incubation with 5-ALA, OAT, SW1710 and normal epithelial cells have shown approximately the same results. This measurements are correlated

with the relative adherence of oral microorganisms to the cells. ALA induction of porphyrin biosynthesis can be achieved in, most species of bacteria, regardless of whether they belong to the Gram positive or the Gram negative division. Even the incubated cell samples without ALA revealed high fluorescence. The relative adherence of bacteria to oral epithelial cells differentiated by acridinorange / ethidiumbromide staining. This method provides an opportunity to examine the relation between amount of oral epithelial cells, and the fluorescence intensity of cell samples. Tongue cell biopsies, revealed more intensity than cheek cell biopsies. And the good oral hygiene revealed less intensity than the poor hygiene. And we have been proved beyond any doubtful the role of bacteria in restriction of specificity and false-positive results in and ex-vivo. Furthermore, because of its large variation between both anatomical locations and volunteers, the total fluorescence intensity can not easily be used as a method of diagnosing tumour tissue. Some studies have investigated whether the use of a reference database improves the sensitivity and specificity of early cancer detection, some locations are interchangeable for the purpose of autofluorescence spectroscopy. And this is not the case in our experiments where the cell cultures and cell suspension are used. This is one of advantages of photodiagnosis of oral cancer ex-vivo. Although successes have been obtained in diagnosing malignancies in other organs, we believe that this method is not enough reliable for diagnosis of the oral cancer in-vivo because of the possibility of false positive results.

4.3 Early Detection of Oral Cancer by Brushing Biopsy

Collection of oral transepethelial cells by brushing of the oral mucosa is called ´´oral brush biopsy``. The oral biopsy coupled with computer-assisted analysis, has been developed as an alternative technique for evaluating the alteration of the surface epithelium of oral mucosa. Lesions that require brush biopsy include unexplained clinically detectable alterations of the surface epithelium, whether cancer or precancer is suspected. But there are some reports of significant rates of false negatives from brush biopsy. Potter et al. [95] examined all diagnosis of oral squamous cell carcinoma from a university oral pathology service over a two-year period, to determine if there is any negative for epithelial abnormality. Further investigations are done to definitve tissue diagnosis. Potter found 4 of 115 squamous cell carcinomas that were reported to be negative on brush biopsy, a false negative rate of 3.5 percent. A 3.5 percent false-negative rate may be acceptable, particularly if one compares this result with a screening modality like mammography, which has a false-negative rate that varies from approximately 6 percent to 25 percent. This may (also) delay diagnosis if the results are

negative. If the results of brush biopsy are negative, the diagnosis might be wrong. And this making it difficult to determine appropriate treatment or whether an additional procedure is necessary for diagnosis. This results are needed for two procedures, rather than one, to establish a diagnosis. The need to perform two procedures may significantly delay diagnosis. In the study described above, Potter reported an "undeniably unacceptable" average delay in diagnosis of squamous cell carcinoma of 117.25 days (range 5 to 292 days) with oral brush biopsy. The investigators stated that this delay "can be potentially disasterous" [61]. Oral brush biopsy has been criticized for adding time and cost to the diagnosis of oral lesions without additional benefit to the patient. Because the brush biopsy detects only cellular atypia, positive oral brush biopsy results must be confirmed with a scalpel biopsy for definitive diagnosis. There is insufficient evidence to support the use of oral brush biopsy as a general screening technique for oral lesions. The National Cancer Institute, the Canadian Task Force on the Periodic Health Examination and the U.S. Preventive Services Task Force (2004) have recommended against routine screening for oral cancer using a brush biopsy [96]. A Cochrane evidence review found that there is no evidence from prospective clinical trials that screening with brush biopsy reduces mortality [97]. On the other hand many studies support brush biopsy as a method for early diagnosis of oral cancer, in spite of prescence of false results [32-40]. Some of this studies have attributed this false results to: clinicians, in most cases are responsible for false negative cancer diagnosis (sampling error). Due to non representable acquisition of cells or tissues [36]. In gynaecological and pulmonary cytology this error accounts for about 80% of false negative diagnosis. Errors due to microscopically overlooked tumor cells by cytopathologists are less frequent than due to sampling errors [98]. The explanation of other false negative cases is due to geographic error during taking the smear when the lesion is located in area with diffcult access [35]. Furthermore, the number of collected cells could be responsible for bad sensitvity and errors if its not high enough [38]. The false negative results have been observeded in histology as well, that could lead to delayed of diagnosis or more surgery in case of positve tumor cell diagnosis and negative histologic follow-up patients [37]. Despite the success of cytologic and histologic investigations have contributed greatly to the fight against malignant disease worldwide, the diagnostic accuracy of these methods in pathology still has its limits. It is important to improve the brush biopsy with other techniques to increase the reliability of this method, as a diagnostic tool for oral precancer and cancer lesions.

4.4 Combination of Cytological and Photodynamic Diagnosis

4.4.1 Suppression of bacterial porphyrin during PDD In-vivo

The 5-ALA -based fluorescence diagnosis showed a specificity of approximately 62 %. This specificity was mainly restricted by tumor detection in oral cavity in the prescence of bacteria. After topical ALA application in the mouth of patients with previously diagnosed oral sequamous cell carcinoma (OSCC), red fluorescent areas were observed which did not correlate to confirm histological findings [74]. In many studies, fluorescence was investigated from different bacteria strains found naturally in oral cavity after ALA incubation, most of strains produce large amounts of porphyrins, mainly protoporphyrin IX [71-75]. We have also proved in this study the ability of oral microorganisms to produce huge amount of PpIX when incubated with 5-ALA. Moreover the bacteria are able to produce PpIX without exogenous induction of 5-ALA, with significant increase in fluorescence. These results clarify that PDD may lead to false positive findings if we did not suppress the effect of bacteria fluorescence. There are many antibiotics and antiseptics can suppress the bacterial activity. The problem was the way of using this antiseptic in oral cavity before starting the procedure. Some studies used PBS as a mouth wash to eleminate the bacteria and reduce fluorescence. We approved beyond any doubtful that the use of PBS has not any effect on the ability of bacteria to produce PpIX. There is many experiments tried to diminish the 636-nm peak intensity. Rinsing the mouth with saline had no effect on the height of the peak at the tongue, but rubbing the tongue could reduce the 636-nm peak to about 10 30 % of its original height (fig. 3.21). If all of the fluorescence producing substances were located in the tongue tissue cells, it would have been impossible to diminish the porphyrin-like peak. The using of other antiseptic materials may not lead to significant reduction in bacterial associated fluorescence. Other strong antiseptic like chlorhixidine (synthesized at the end of 1940) is an antimicrobial agents seek to either kill microorganisms or control their growth by extending the lag phase as long as possible. Such agents may not be as active in vivo as laboratory studies would suggest: some rely on growth of the organisms to be effective (eg. penicillin requires active cell wall synthesis) and so are less effective against slow growing organisms in vivo, and organisms within bio-films are protected from external agents [99]. The chlorhexidine needs around 30 minutes to start releasing after binding to oral surface from where its released gradually into saliva over many hours at bacteriostatic concentration [100]. The prescence of saliva may reduce the effective of chlorhexidine particularly the used concentration is low because of toxicity of chlorhexidine to human cell and tissues [101]. The action of chlorhexidine is by

adsorbing onto the cell wall of microorganism and causing cell death, this action demands a certain period of time according to the strain of microorganisms, espicially for some microorganisms that are much more resistant. So the use of chlorhexidine within limited time seems to be indeterminate to significant reduction in bacterial fluorescence. How ever keeping the chlorhexedine in oral cavity or washing several times before start the procedure is not practical. In the cases where the oral hygine level is high, furthermore the regular use of chlorhixidine lead to significant reduction in bacterial fluorescence (fig 3.21).

4.4.2 Suppression of bacterial porphyrin during PDD ex-vivo

Exfoliative techniques have the advantage of being minimally invasive, and they do not require local anesthetic. Use of cytobrush reportedly allows sampling of the full thickness of stratified squamous epithelium of the oral mucosa. Caution in using this technique is recommended for several reasons (reports of false-positive and false-negative results) have been posted on the web site of Bulletin Board for oral pathology [1]. In this study we have collected oral epithelial cells from 37 healthy adults by brush, 50 samples have prepared immediately and incubated. Different methods were done to decrease the effect of bacterial fluorescence in common with other studies. In the case of the using of PBS as a mouth rinse before cell collection, a higher PpIX fluorescence intensity was mostly achieved in most of samples. On the other hand we used PBS to wash the samples that have been prepared from various oral hygiene volunteers, a similarity was found among the of the PpIX fluorescence kinetics in most of 5-ALA treated samples. The PpIX fluorescence kinetics in 50 % of samples are gradual decreased after washing, and in 30 % of samples did not reveale any changes in PpIX fluorescence, whereas the fluorescence disappeared in other samples. One of the aims of this work was to assess the pharmacokinetics of 5-ALA-induced PpIX fluorescence in healthy oral epithelial cells as well as in tumor cells ex-vivo. There are no significant results achived by sample washing for this assessment, the samples presented differences in the fluorescence from one sample to another, which means PpIX accumulation is quite heterogeneous in the washed samples. The further investigation that have done for the same samples, was under fluorescent microscope: in samples with high fluorescence, the epithelial cells and bacteria were enough to emit fluorescence. These amount of microorganisms is responsible for the high fluorescence. The samples that revealed reduction of fluorescence, contain more less bacteria and this might be explained by a higher oral hygiene of volunteers. In some samples we observed disappearance of fluorescence compared to the other samples, and that was due to elmination of most of cells and bacteria during

washing. Our technique (washing by PBS and centrifuge three times) that have been used for disposition of bacteria from epithelial cells, showing unsuitability for suppression of bacterial fluorescence effect by elimination of bacteria, whereas the bacteria were still adherent to epithelial cells. Oral epithelial cell variability for bacterial adherence is confirmed in other studies either. Different techniques were used to examine the relation between oral epithelial cell type and oral candidosis in specific groups, such as tobacco smokers, where increased epithelial cell keratinisation and candidal colonisation has been reported [101-102]. Equal volumes of oral epithelial cells and candida were mixed and incubated. The epithelial cells from this mix were collected by filtration through 10 microns polycarbonate membrane filters. Cells were stained and examined, candida albicans had a higher level of adherence ($p < 0.001$) to oral epithelial cells, mean (SD) number of candida attached to 100 oral epithelial cells 562 [101]. Our results confirmed the role of bacteria that are adherent to oral epithelial cells in porphyrin fluorescence. Other technique was used in other study [102], the flow cytometric assay is used as a tool in the analysis of oral streptococci adherence to oral epithelial cells. There is a ~60 fold increase in the MFI (mean fluorescence intensity) of cells incubated with bacteria compared to the epithelial cells alone fig 4.1 [102]. With flow cytometric assay, there is a statistically significant difference between the strains in adhesion to squamous cell carcinoma (SCC). And once more, the study confirmed our clarification of the higher autofluorescence of malignant ulcer due to SCC compared to normal ulcer.

More effective methods for bacterial fluorescence suppression are needed. In second step of our investigations, the chlorhexidine and the tetracycline have been investigated. An advantage of 5-ALA are the spectral properties of PpIX. Despite the tetracycline is broad spectrum antibiotic, we have put a side the tetracycline due to the strong fluorescence, that interferes with the PpIX fluorescence in comparison to chlorhexidine that has only very weak auto-fluorescence (fig 3.23). The using of chlorhexidine as a mouth wash before cell collection by brush showed various results, and these results depended on the oral hygiene in addition of other situation like the dental caries and periodontitis. And under these conditions, the type and amount of bacteria will change. A highly significant difference between the maximum PpIX fluorescence intensity was found. We have investigated two groups, one group used PBS as a mouth rinse, for other group the mouth rinse was the chlorhexidine. There were restricted significant results for second group in comparison to PBS group, and these results were directly proportion with the situation of the mouth and the levels of oral hygiene that have been illustrated in 3.5.3. The over all result from these investigation is that, using antiseptic agents as a mouth rinse to suppress the bacterial fluorescence; seems to be

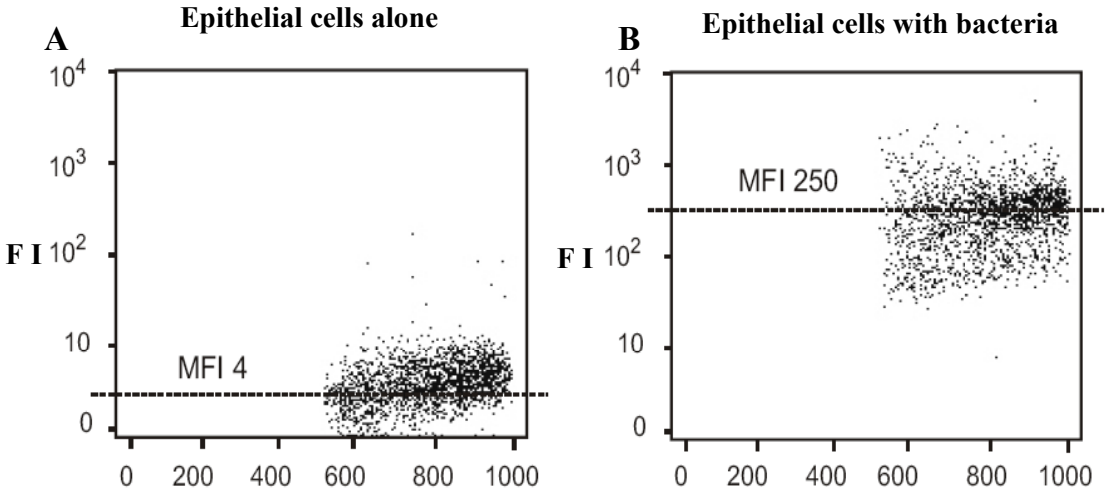


Fig 4.1 Flow cytometric analysis of human epithelial cells alone and after incubation with oral streptococci. (A) Autofluorescence of the SCC cells in dot plot. (B) Fluorescence of the SCC cells after incubation of the cells with labelled bacteria. (MFI, mean fluorescence intensity). [102]

ineffective or very restricted to the high level of oral hygiene with regular use of antiseptic agents. Our findings suggest that 5-ALA with chlorhexidine as a vehicle seems to be a promising molecule for fluorescence diagnosis of tumor cells. This effect has been already demonstrated for the topical administration of chlorhexidine with 5-ALA when compared to the results by tetracycline. It would be important to investigate the cytotoxicity of CHX at low concentrations in cell lines derived from oral tissues and to correlate these findings with a potential anti-microbial activity. Thus, it was the goal of this investigation to evaluate the CHX concentrations which decisively inhibit growth and survival of bacteria. In addition, whether those inhibitory concentrations are cytotoxic as well should be investigated by different tumor cells as well as oral epithelial cell that are collected by brush and HEK 293. The various concentrations of chlorhexidine gluconate were added to cell suspension, and the bactericidal effects of both 0.05, 0.01 % and 0.2% CHX were detectable at 120 min. Our data revealed that chlorhexedine inhibits proliferation of oral microorganisms even at low concentrations while a concentration ≥ 0.2 % of CHX are cytotoxic to both cells and bacteria (fig 3.26). All samples that have been treated with CHX, are subjected to microscopic investigation to determine the percentage of viable cells. While total fluorescence measurements were capable to measure the antimicrobial effects of CHX at a concentration of 0.05 % and 0.1 %, total fluorescence measurements for the samples exhibited barely detectable at a concentration of 0.2 % CHX. Due to the lethal effect of this concentration on microorganisms and their host. We have shown that lethal concentration of chlorhexidine on cell samples is 0.2 %, and the sublethal concentration was 0.01%. whereas 0.05% CHX has

no significant effect on the host (epithelial cells) and time-dependent bactericidal effects (approximetly 30 min before ALA application). This concentration has lethal effect on some kind of microorganisms like C. Albicans, where a concentration of 0.004% chlorhexidine gluconate was sublethal for actively growing of C. Albicans [103-104].

A fall-off in the overall fluorescence intensity evaluated at 635 nm was obtained for all the oral epithelial cells samples, that have been. treated with CHX, as well as an increased in the fluorescence intensity at 653 nm (PpIX peak) in all malignant samples that have been treated with CHX and 5-ALA. The lower CHX concentration seems to be sufficient to obtain a useful diffrentiation ratio between normal and malignant cells with a very low PpIX fluorescence intensity in the normal cells. A PpIX fluorescence maximum seems to occur between 2-3 hours in normal tissues, whereas malignant tumour tissue shows a higher PpIX fluorescence level for a longer period of time.

4.4.3 PDD of oral cancer: ex-vivo fluorescence cytology using CHX

Because of the large variation between both anatomical locations and volunteers, the total fluorescence intensity can not easily be used as a method of diagnosing oral tumour lesion. Some investigations have planed to use reference database for comparison [85]. Different anatomical locations showed significantly different autofluorescence intensities. However, the interpatient variability was much larger. They presume that because of large anatomical variation, therefore they can not used the fluorescence intensity as adignosis when using a reference database. Hence, there is no imrovement of sensitivity and specificity of early cancer detection in-vivo by using of a reference database. The data from our in vitro study has shown that 5-ALA induced fluorescence can be used for photodynamic diagnosis (PDD) in different human cancer and normal cells. According to previous study that have Performed by our group [67], the results shown that the application of 2 mM 5-ALA could provide sufficient PpIX fluorescence for reliable photodetection of malignant and normal cells. All the our investigations ex-vivo are done at the same excitation wavelength (405 nm) and measured in the red spectral region [105]. We could also observe a clear contrast between malignant cells (OAT, SW1710) and other cell lines (HEK293), furthermore the normal oral epithelial cell. Compared to our group´s previous 5-ALA-induced porphyrin fluorescence spectroscopy study, we achieved results demonstrated the ability of fluorescence spectroscopy, to differentiate between minimum number of malignant cells and oral epithelial cells ex-vivo. Our results have confirmed additional measurements that have done by optical probe. The (SD) of single measurement 1-4 %, 1-3 % for 100 and 500 malignant cells respectively. The

results confirm that the red autofluorescence (636-nm peak) which can be found in oral squamous cell carcinomas originates from fluorescent bacteria fig 4.2 [106], and not from enzymatic defects in heme synthesis. High concentrations of these bacteria can be found in necrotic and ulcerated tumor lesions. 5-ALA induction of porphyrin biosynthesis can be achieved in most species of bacteria, regardless of whether they belong to the Gram positive or the Gram negative division [64, 72]. Thus, red autofluorescence does not seem to be an appropriate method for the detection of early-stage squamous cell carcinoma, whereas the probability of false positive result is very high.

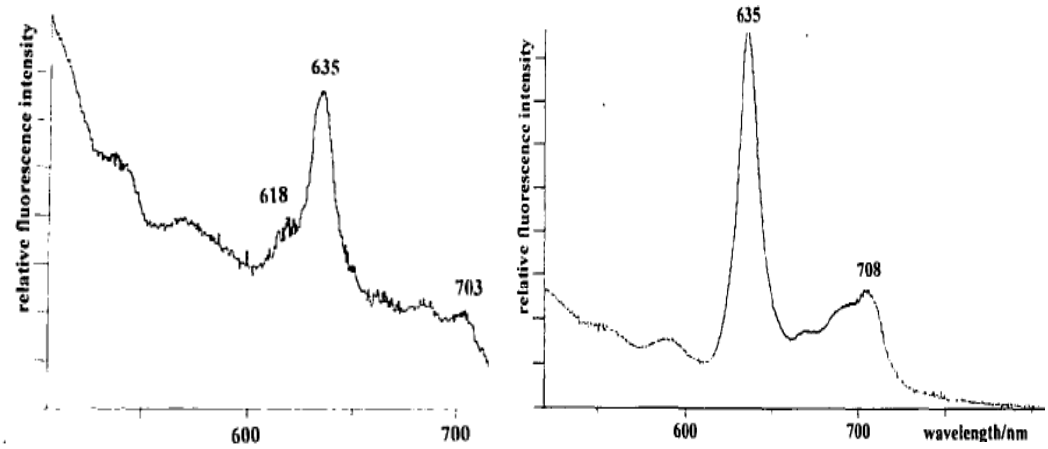


Fig 4.2 Autofluorescccnce spectrum of the gram-negative bacterium (Left) pseudomonas aeruginosa, (Right) bacteroides intermedius [106].

There is many experiments tried to diminish the 636-nm peak intensity. Rinsing the mouth with saline had no effect on the height of the peak particularly at the tongue, but rubbing the tongue could reduce the 636-nm peak to about 30 40% of its original height [85]. If all of the fluorescence producing substances were located in the tongue tissue cells, it would have been impossible to diminish the porphyrin-like peak. We have examined the relative adherence of bacterial organism to oral epithelial cells that have been collected by brush, before and after washed by PBS and centrifuge. It is well known that oral mucosal epithelial cells contain significant numbers of bacteria which are so firmly attached to their surface that they cannot be removed by vigorous washings [107]. Under fluorescence microscope, we observed huge number of bacteria that have been adherent to squamous epithelial cells in different locations of tongue and less number of bacteria adherent to cheek. Our results confirm and expand the findings of previous investigations that showed, the different strains of S. salivarius, S.sanguis, and S. Mutans, have more ability to attach to tongue and cheek surfaces in the oral cavity table 1.

Table 1. Adherence of Streptococcus salivarius, S. santguis, anid S. mutanis to oral epithelial cells [73]

Organism	Avg. no. of bacteria/epithelial cell	
	Human cheek	Human tongue
Avg. background control	19 ± 6[a]	111 ± 17
S. salivarius 9GS2	282 ± 6	
S. salivarius 10G3	101 ± 13	229 ± 15
S. salivarius 1A		
S. salivarius SS2	99 ± 14	
S. sanguis 34	83 ± 12	190 ± 12
S. sanguis A15	160 ± 17	
S. sanguis H20	55 ± 9	
S. sanguis H10	120 ± 14	
S. sanguis H7P		
S. mutans 6715	56 ± 13	
S. mutans GS5	19 ± 3	
S. mutans HS6	46 ± 9	

[a] Calculated standard error of mean.

The elmination of bacteria to reducing their effects in PDD, are not possible. The results of our experiments are revealed that, CHX at concentrations ranging from 0.05 to 0.1 % affect the growth of bacteria. Further, CHX was not cytotoxic to the different cell lines. while concentrations between 0.2 % completely inhibited the growth of the bacteria with cytotoxic effect on host cells. Thus, our data clearly indicate that chlorhexidine is a very effective growth inhibitor of bacteria, even at very low concentrations which are not cytotoxic in human epithelial cell at the same time. Exposure to CHX does not kill bacteria immediately; a delay of 20 seconds has been reported for different straines of bacteria. In this study with oral bacteria that have been attached to epithelial cells, the bactericidal effects of 0.2% CHX were detectable at 3 to 5 min, and that was coincided with other studies, and for 0.05 and 0.1 % CHX at 30 min [108]. All the fluorometric measurments are confirmed our findings, the 405-nm excitation wavelength produced the peaks of 5-ALA and CHX treated normal samples, in the red region at 635-nm, which, when used in conjunction with the higher peak intensities in the red region of the neoplastic cell samples (treated by 5-ALA and CHX), povide an excellent signature for sbnormal samples. The results of this study are important because they demonstrate the the diagnostic potential of fluorescence spectroscopy for the

diagnosis of lesions of the oral mucosa. However, the data presented in this report were obtained from a small number of volunteers. The fluorescence maximum of porphyrin secondary to bacterial synthesis within oral tissue. Several bacteria, such as Escherichia coli, Klebsiella pneumonia, and Staphylococcus pyogenes, among others, are known to produce porphyrin and to induce red fluorescence in ulcerated tissue.

Any method developed for analysis in-vivo should take into account that the patient-to-patient variation of healthy volunteers is great and that the variation within a particular location in each person is small. Thus, any comparison of normal to abnormal tissues should focus within a single patient. The need exists for further study with more patients and volunteers to develop diagnostic algorithms that can adequately differentiate between normal, inflammatory, premalignant, and malignant oral mucosal tissue. More effective methods for early detection of oral cancer are needed. Many studies proved the possibility of diagnosis of suspicious lesions ex-vivo with high reliability [67-74-80-109]. Moreover, in the ex-vivo, it was possible to observe that the fluorescence intensity induced by 5-ALA can be much reliable than fluorescence in-vivo, in the abscence of specific effectiveness of alterations in tissue architecture or bio-chemical composition that causing the overall differences in fluorescence intensity of tumor and normal tissue have not been elucidated. And Because human tissues contain several kinds of fluorophores and each of fluorophore has its own excitation-emission character. Further more the natural fluorophorres that may undergo changes in quantity or form during tumor progression include flavins, NADH, collagen. This might facilitate the scanning of large number of cells in different samples to avoid more diagnosis procedures and also reduce costs in the clinical application. Fluorescence spectroscopy is non-invasive, rapid simple and reproducible. Easy to handle and to evaluate the findings. On the other hand, the brush biopsy is non-invasive, cheap, simple chair side procedure and painless procedure can be done without local anaesthetic, which can provid intact cells from different layers of epithelium. We have been developed a technique for evaluating the lesions of the surface epithelium of the oral mucosa. The oral brush biopsy coupled with fluorospectrometric analysis. The system is suggested to be a useful tool for immediate diagnosis of oral cancer. Our experiments revealed that, by means of an optical microprobe, very few cancer cells (100) can be detected. Adding chlorhexidine before incubation of brush biopsies with 5-ALA increases reliability of the test by largely reducing the fluorescence signal due to the presecence of bacteria. Chair side diagnostics of epithelial carcinoma seem feasible. This might facilitate the scanning of large tissue areas and reduce costs in the clinical application.

5 Summary

The WHO reported oral cancer as having one of the highest mortality ratios amongst all malignancies. The death rate associated with this cancer is particularly due to the cancer being routinely discovered late in its development. Early detection is essential to determine the strategy of treatment and to improve the prognosis of oral cancer. During the past few years, many screening strategies for improving oral cancer mortality have evolved as promising technologies. PDD and cytological studies of cells are non-a aggressive techniques, well accepted by the patient and, therefore an attractive option for the early diagnosis.

The in vitro study presented here has two main objectives:

(1) to test the hypothesis that auto-fluorescence spectroscopy after 5-ALA application can differentiate very few normal cells (100) from diseased cells.

(2) to investigate whether the combination of PDD with oral brush biopsy might become suitable chair side tool to detect early oral carcinoma.

Small numbers (100-500) of established human tumor cells (small cell lung carcinoma, OAT 75; transitional cell carcinoma of the bladder, SW1710; HEK 293) were incubated with 2 mM 5-ALA. In addition, 50 brush biopsies from 37 volunteers (with different ages 18-45 years old and oral hygiene) have been prepared. After two and three hours of incubation all samples were investigated by means of spectrofluorometry. Measurements were performed in capillaries. For excitation (405 nm) and detection of fluorescence spectra a 400 μm fibermicroprobe–microspectrometer system was used.

A minimum of 100 malignant cells and three hours of incubation with 5-ALA are needed to detect a typical spectrum for PpIX. Some epithelial samples from brush biopsy showed strong (bacteria related) PpIX autofluorescence which increased after adding 5-ALA. From testing various antibiotics and antiseptics it emerged that 0.4 mM chlorhexidine strongly reduced fluorescence in brush biopsies (containing only healthy cells), whereas the fluorescence signal of established cancer cell lines decreased only a little. The ex-vivo experiments revealed that, by means of an optical microprobe, very few cancer cells (100) can be detected. Adding chlorhexidine before incubation of brush biopsies with 5-ALA increases reliability of the test by largely reducing the fluorescence signal due to the presence of bacteria. which, when used in conjunction with cancer cells, provide an excellent signature, this difference was statistically significant ($p<0,05$). Chair side diagnostics of epithelial carcinoma seem feasible.

6 Zusammenfassung

Laut Berichten der WHO hat Krebs in der Mundhöhle eine der höchsten Krebssterblichkeiten. Die hohe Mortalität beruht auf der Tatsache, dass diese Tumorformen meist erst in einem fortgeschrittenen Stadium diagnostiziert werden. Früherkennung aber ist essentiell, um ein geeignetes Behandlungsschema festzulegen und die Prognose bei einer solchen Erkrankung zu verbessern. In den letzten Jahren wurden viele vielversprechende Screening-Methoden entwickelt, mit dem Ziel die Mortalität zu senken. Die Photodynamische Diagnose (PDD) und zytologische Verfahren sind schonende Verfahren, die von den Patienten gut angenommen werden und sind daher attraktive Alternativen für eine Früherkennung.

Die hier vorgelegte in-vitro Studie hat zwei Themenschwerpunkte:

(1) Können nach Inkubation mit 5-ALA mit Autofluoreszenz-Spektroskopie in einer Probe mit nur wenigen Zellen (100) normale von malignen Zellen unterschieden werden?

(2) Ist die Kombination von PDD und Bürstenbiopsien aus der Mundhöhle eine geeignete "Chair Side" Methode für die Früherkennung von Tumoren der Mundhöhle?

Eine kleine Zellzahl (100-500) etablierter menschlicher Tumorzellstämme (Small Cell Lung Carcinoma, OAT 75; Transitional Cell Carcinoma der Blase, SW1710; HEK 293) wird dazu mit 2mM 5-ALA inkubiert. Zusätzlich werden 50 Bürstenbiopsien von 37 Probanden (Alter 18–45 Jahre, unterschiedliche Mundhygiene) präpariert. Nach zwei und drei Stunden Inkubation werden alle Proben spektrofluorometrisch untersucht. Die Messungen finden in Glaskapillaren statt. Für die Fluoreszenzanregung bei 405 nm sowie für das Erfassen der Fluoreszenzspektren wird ein 400 µm Fasermikrosonde–Mikrospektrometer verwendet.

Mindestens 100 maligne Zellen und drei Stunden Inkubation mit 5-ALA sind nötig, um ein typisches Protoporphyrin IX-(PpIX)-Spektrum zu detektieren. Einige Proben der epithelialen Bürstenbiopsien zeigten eine starke bakteriogene PpIX-Autofluoreszenz, die durch die Inkubation mit 5-ALA noch verstärkt wurde. Verschiedene Antibiotika und Antiseptika wurden getestet. Es zeigte sich, dass 0,4mM Chlorhexidin die bakteriogene Autofluoreszenz in Bürstenbiopsien stark reduziert, während das PpIX-Fluoreszenzsignal in den Tumorzellinien nur geringfügig reduziert wurde. Diese ex-vivo Experimente zeigen, dass mit der "Optischen Microsonde", eine sehr geringe Zahl (100) maligner Zellen nachgewiesen werden kann. Der Zusatz von Chlorhexidin zu den Bürstenbiopsien erhöht die Zuverlässigkeit des Tests, da die bakterielle PpIX-Autofluoreszenz reduziert wird. Die Fluoreszenzintensität von Tumorzellen unterscheidet sich davon statistisch signifikant ($p<0,05$). Eine "Chair Side" Diagnose epithelialer Tumoren der Mundhöhle scheint realsitisch.

7 Abbreviations

CHX	chlorhexidine
CT	computed tomography
5-ALA	5-aminolevulinic acid
HpD	hematoporphyrin derivative
MRI	magnetic resonance imaging
NSCLC	non-small cell lung cancer
OSCC	oral squamous cell carcinoma
PBS	phosphate buffer solution
PS	photosensitizer
PDD	photodynamic diagnosis
PDT	photodynamic therapy
PpIX	protoporphyrin IX
RBG	red/ blue/ green
SCLC	small cell lung cancer
TCC	transitional cell carcinoma
TNM	tumor / lymph nodes / metastasis
UC	Urothelial carcinoma

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Work experience

2000-2004	Assistant at Oral Biology and Oral Diagnosis Departments. Dentist at Polyclinic, Tripoli and in private Clinic, and as a Teacher of Basic Science at Primary School.
2004	Scholarship to continue promotion studies in Oral medicine in Germany.
2005-to date	PhD student in Institut für Lasermedizin, UniversitätsKlinikum Düsseldorf, Germany
2007	Poster A ward (First Prize) for the Scientific, Paper Ex-vivo PDD to detect malignant cells in oral brush biopsies, presented at the 4th Congress of the Society for Oral Laser applications SOLA 2007, Bruges, Belgium.