Applications of Confocal Laser Scanning Microscopy in Dentistry.
Study of the changes of the post-extraction sites.

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1. Introduction

The Confocal Laser Scanning Microscope (CLSM) is an optical microscope that includes a laser light as a light source and an electronic system which helps on image processing. It obtains high-resolution and extremely thin optical image sections, removing the interference caused by the light arriving from the different optical fields across the thickness of the sample, and focusing on a single plane (confocal). Therefore, since the images obtained are digital images, unsuspected magnifications for optical microscopy can be obtained.

The CLSM works as follows: it removes the veil that is generated in the regions that are placed outside the focus plane in a normal optical microscope image. The incident light on the sample passes through a small hole or slot (pinhole) and focuses on the image plane of a high numerical aperture objective, so that the light reflected by the point which is placed in the focal plane of the objective returns to it and is refocused and transmitted by a pinhole without any loss. By contrast, the light scattered or emitted by the points outside the image plane is dimmed or completely blocked. Thus, an image of high contrast and definition of a point on the focal plane is obtained without being affected by the regions which are out of focus. As the apertures of both the lighting and the return of the image have a common focus, this kind of microscopes have been called Confocal Microscope. In summary, Confocal Microscopy is based on an improved relationship between the signal and image noise.

Fig. 1 Schematic diagram of the optical pathway in a CLSM system [1]. After passing through a first pinhole, the laser light is projected into the specimen by the dichroic mirror and the objective lens. The light emitted from the sample is collected by the objective lens and passes through pinhole 2. This pinhole discards rays that are reflected by out-of-focus planes.
Figure 1 shows a schematic diagram of the optical pathway in a CLSM system. The light beam passes through pinhole 1 and comes in contact with a dichroic mirror, which entirely reflects the incident light with an angle of about 45 degrees. Then, the light beam is focused onto the sample using the microscope objective. The light emitted by the sample is collected by the same objective and focused on detecting pinhole 2, passing through the dichroic mirror. The light coming from places located at a greater or lesser depth in the sample (out-of-focus planes) comes into contact in front of or behind pinhole 2. Because the amount of incident light on the sample is extremely small, it is necessary to use powerful light sources such as laser beams.

The procedure gives us the image of a small point in the sample. For a complete picture it is necessary to use complex procedures to move the point of illumination across the sample, and integrate this image of individual points into a single image. Systems that allow displacing the sample or moving the point of light are used, scanning the entire area that is wished to analyse. This kind of microscopes are called Confocal Laser Scanning Microscopes. To build an image it is necessary to cover the entire sample uniformly. In addition the illuminating beam and the return pathway must be perfectly aligned.

Regardless of the procedure used to scan the sample, CLSM images are notably higher than those obtained with the conventional optical microscope because the generated images contain volumetric and texture details impossible to obtain with the conventional ones.

Confocal Microscopy also allows the study of the specimens using transmitted or reflected light. This means that samples that are not transparent due to thickness or nature can be analysed. New techniques for the analysis of specimens have been developed, which do not involve cutting them into thin slices.

The main advantages of CLSM are [2]:
1)Higher resolution. Resolution increases with the shorter wavelengths and is higher as the numerical aperture of the objective increases.
2)Greater contrast. The veil that produces regions outside the in-focus plane is removed.
3)Possibility to obtain optical slices. By changing the pinhole aperture and the focal plane it is possible to obtain slices of different planes.
4)Three dimensions reconstruction. With the slices obtained in the different focal planes, it is possible to create a 3-D image of the specimen under study.
5)Image analysis. It is possible to digitalize the image and use imaging techniques as morphometric measures.

2. Applications of Confocal Laser Scanning Microscopy in Dentistry

The Confocal Laser Scanning Microscope has been used in the field of dentistry for the evaluation of new restorative materials in dental therapy or the determination of the bone-implant interface. The first papers which described the use of CLSM in dentistry were published in the 90's [3-5]. Since then, CLSM has been used in many dental studies. Here are some of CLSM applications:

2.1 Applications of CLSM in dental therapy

The long-term stability of resin-composite restorations depends on the resultant structure of the adhesive interface created between restorative materials and dental hard tissues [6]. Current dentin bonding techniques depend upon development of an interfacial layer created by the diffusion of low-viscosity resins from dentin adhesive systems into demineralised dentin by treatment with phosphoric acid or structurally modified by highly acidic, self-etching dentin primers. This resin-impregnated dentin layer extends only a few micrometers into dentin and has been referred to as the hybrid layer [7]. Submicron hiatus represents a potential space between the base of the collagenous network and the mineralized dentin when it is acid-etched for bonding. CLSM has been used to characterize the resin-dentin interface of different restoration materials [8-17]. CLSM allowed measure width of the space without destroying the samples [18]. When submicrometer hiatus structures are examined using CLSM and Scanning Electron Microscopy (SEM), CLSM maintains specimens under constant humid conditions, as opposed to the typical drying artifacts commonly seen with SEM specimen preparation techniques [19].

Enamel and dentin surfaces treated with acid-etched has been observed to determine the effectiveness of different materials [20-25]. Also, resin penetration in root canals filled with different substances can be measured too [26-28].

CLSM has also been used to characterize the effect of different dental procedures on healthy and pathological dental tissues. Therefore, effect of bleaching agents on normal enamel [29-34] and enamel with early artificial caries lesions [35, 36], surface analysis of enamel and dentin after Nd:YAG laser [37], Er: YAG laser [38] and CO₂ laser [39, 40] irradiation, dentin tubule occlusion with a desensitizing dentifrice [41, 42], improvement of the remineralization effect of topical fluoride using iontophoresis [43], caries removal effectiveness of dentin excavation methods [44] and root resorption related with orthodontic forces [45] have been investigated.

Another line of research where CLSM is being used is the study of dental wear and erosion of normal dental tissues [46-48], white spot lesions [49] and different restorative materials [50-52].
Caries formation is one of the major reasons for failure and replacement of restorations. The changes generated in the dental tissues due to carious lesions have been observed by CLSM. The depth of caries lesions produced by biological and chemical artificial models in permanent and primary dentin has also been analysed [53], as well as surface changes [54, 55], mineral content [56] and enamel remineralization [57]. When caries development is examined by microradiography (TMR) and CLSM, initial enamel caries not detected by TMR could be visualized by CLSM [58].

CLSM has also been used to assess the cariostatic effect of different restorative materials or different fluoride compounds. Inhibition zone formation and mineral distribution along the interface of adhesive systems containing fluoride or antibacterial primer or not, after chemical and biological artificial caries challenges have been evaluated [59]. Inhibition of lesion progression by the penetration of resin [60-63] or fluoride varnish treatment [64, 65] has been studied too.

2.2 Applications of CLSM in bone analysis

The first studies that analyzed the bone by CLSM were performed in the field of orthodontics, where alveolar bone remodeling with orthodontic tooth movement and retention was investigated [66].

To define the value of CLSM as a practical method for a qualitative and quantitative analysis of hard tissue normal maxillo-facial bone was analysed [67]. CLSM allowed improved tissue imaging, bidimensional pictures with better resolution at cellular level, and, in particular, the possibility of different histomorphometric evaluation. Besides healthy bone, pathological bone has also been studied. Histological and histomorphometric differences in bone structure in patients with bisphosphonate-related osteonecrosis of the jaws and in healthy patients have been evaluated [68]. Bisphosphonate-related osteonecrosis are jaw lesions, mainly consisting in bone necrosis. CLSM allowed the histological analysis of bone and obtaining the histomorphometric parameters.

Another field where CLSM has been widely used is in dental implants. The interface between the transmucosal portion of endosseous implant surface and the connective tissue is characterized by fibroblast-rich barrier tissue, which is important for the long-term stability and maintenance of the implant [69]. CLSM allowed to examine biological behavior of fibroblast-like cells. Bone integration of titanium implants is modulated by its surface characteristics. Histomorphometric evaluation of the bone around a new implant surface treatment by CLSM has been carried out in many studies [70-73].

Mechanical properties of bone are greatly influenced by the percentages of organic and mineral constituents. It is essential to accurately characterize the implanted surfaces. Therefore, bone mineral density of peri-implant bone has been assessed under scanning electron microscopy with backscattered electron signal (BSE), light microscopy (LM) with a double staining technique, fluorescence microscopy and CLSM. Under CLSM the same sections showed the area of bone modelling closest to implant surface [74]. The observation of bone formation in undecalcified sections with titanium implants at high magnification was very difficult because of the complication of sectioning bone together with implants. CLSM allows to examine both the implant surface and adjacent bone [75]. It also permits analyzing tissue-implant interface with non-invasive conditions [76].

CLSM has recently been used to perform micromorphological and quantitative roughness analyses of osteotomized bone surfaces of different ultrasonic osteotomes [77]. The ultrasonic osteotome procedure is an alternative to conventional methods of osteotomy in oral and maxillofacial surgery.

2.3 Applications of CLSM in dental hard tissues analysis

CLSM has allowed to visualize the characteristics of enamel, dentin and dental cement, and the distribution of its components. Distribution of collagen in the coronal dentin [78] and lipid components of an organic matrix in mature enamel [79] have been assessed. The shape and arrangement of enamel rods in human deciduous and permanent teeth has been examined with CLSM and scanning electron microscopy (SEM). Three-dimensional images obtained with CLSM were similar to those obtained by SEM [80].

Regarding the cells that form part of the tooth, cementocytes of human teeth have been visualized [81, 82], as well as the terminal end of the odontoblast process [83-85].

Oral lesions as calcifying cystic odontogenic tumors (CCOT) [86], odontoma [87], dens invaginatus [88] have been visualized using CLSM. CCOT represents a heterogeneous group of lesions that exhibit a variety of clinico-pathologic features. Theses lesions are frequently found in association with, or containing areas histologically identical to, various types of odontogenic tumors, such as complex/compound odontomas [86]. Dens invaginatus is a developmental anomaly caused by the infolding of the surface of a tooth crown before calcification has occurred. CLSM showed structural anomalies of hard tissues, such as a difference in enamel prism diameter, in number and diameter of peripulpal dentinal tubules and in surface and diameter of cementocyte lacunae between dens invaginatus and control tooth [88].

Tetracycline administration has dental side-effects, so the drug should not be administered to children. However, it and its derivatives are often administered to young adults. Root dentine of a tooth from a young adult affected by tetracycline therapy was examined with SEM and CLSM [89]. CLSM showed that tetracycline bands were made up of numerous smaller bands and that peritubular dentine not associated with bands had incorporated tetracycline. The
results confirm that dentine mineralization is affected by systemic tetracycline therapy and that tetracycline can be incorporated into peritubular dentine after mineralization of the primary dentine matrix.

2.4 Applications of CLSM in microbiologic analysis

Bacteria sometimes adhere to certain surfaces in vitro and in vivo and multiply there, forming a thin bacterial mass. Such bacterial colonization, of both pure and mixed bacterial kinds, in the form of a thin film-like structure, has been referred to as biofilm [90]. It has been reported that biofilm forming bacteria are more resistant to antibiotic treatment and that they are even resistant to immunologic attack, suggesting that biofilm formation may be associated with the virulence of microorganisms [91]. Oral biofilms are primary initiating factors of periodontal disease and caries [92]. CLSM makes it possible to analyze live biological samples, even if the samples are thick. The three-dimensional biofilm structure can be reconstructed by CLSM sectional analysis.

First studies about biofilm with CLSM were performed by Zaura-Arite et al. [93], who combined the advantage of CLSM to visualize the plaque non-destructively with a vitality staining technique to assess the immediate bactericidal effect of chlorhexidine on biofilm, and Takenaka et al. [91], who studied artificial biofilms of Pseudomonas aeruginosa at 4, 8, 12 and 24 hours with CLSM fluorescence mode, to elucidate the structure and function of biofilms.

CLSM has also been used to show that sucrose concentration affects the Streptococcus Mutans biofilm strength, total biomass, and architecture [94]. CLSM has been employed as a method for studying intact natural biofilm. CLSM combined with FISH allows simultaneous analysis of the spatial and temporal dynamics of individual members of microbial populations in their natural habitat [95].

Besides the growth of biofilm on the tooth surface [96, 97], it has also been studied on some materials used in dentistry [98, 99] and in periapical lesions of asymptomatic root-filled teeth [100].

Recent CLSM-based studies on biofilm analyze other methods that inhibit film formation [101-112]. Finally, not only bacteria, but also fungi have been studied [113-118].

2.5 Applications of CLSM in periodontal soft tissues analysis

The periodontal ligament consists of cells, connective tissue fibres, matrix, vessels, and nerves. The oxytalan fibre is one of the two connective-tissue fibre components in the human periodontal ligament, the other being collagen fibre. Chantawiboonchai et al. [119] observed the three-dimensional distribution of oxytalan fibres in mouse periodontal ligament using CLSM and attempted to clarify the relation between those fibres and blood vessels.

The reactions of periodontal tissues to the mechanical stimulation of teeth seem to be different in patients of different ages. Changes in the distribution of oxytalan fibres in the periodontal ligament depending on age were studied. CLSM clearly showed that oxytalan fibres in the aged group were relatively more tortuous and complex than those in the control group. This tortuosity and complexity might imply that the oxytalan fibres in aged mice had lost a considerable amount of their original elasticity [120].

3. Changes in the post-extraction sites studied using CLSM

The tooth anchors to the alveolar bone through the collagen fibres which form the periodontal ligament. The alveolar process is a tooth-dependant tissue that develops in conjunction with the eruption of the teeth. The volume as well as the shape of the alveolar process is determined by the shape of the teeth, their axis of eruption and eventual inclination. Therefore, when the tooth is extracted, the alveolar process undergoes atrophy due to the loss of its function [121].

When a tooth is extracted socket healing culminates with the formation of bone tissue. The first stage of socket healing includes the formation of a clot that completely fills the alveolar socket. This clot is transformed into granulation tissue, and a week later, osteoid formation will have begun. After 6 weeks, the marginal portion of the socket presents immature bone islands. All stages of bone regeneration progressed from the apex and the periphery towards the centre and the ridge of the alveolar socket [122]. During healing, a cortical bridge is formed in the entrance of the socket and, finally, the immature bone is remodelled and replaced by marrow and laminar bone [123].

Tooth extraction is one of the most common treatments in dentistry. Resorption of the alveolar process after tooth extraction may compromise the functionality and aesthetics of dental implants and prosthetic restorations.

One of the main problems in the study of alveolar changes after tooth extraction is ionizing radiation generated by the radiographic analysis. Conventional radiographies do not permit three-dimensional studies. Computer tomography (CT) generates three-dimensional images of the alveolar bone and is used for planning the implant surgery. However CT produce ionizing radiation that are harmful to the patient; consequently, CT is not recommended for the study of morphological changes after tooth extraction, because it would expose patients to unnecessary radiation doses, instead, CLSM is a non-invasive and non-damaging technique and absolutely harmless for the patient [1].
3.1 Description of the CLSM used

To study the remodelling of the extraction sites this research group used a CLSM [1]. The confocal laser scanning microscope is an “Olympus LEXT 3100 OLS” with the following features:
1) The wavelength of the laser is 408 nm (Violet Laser Diode).
2) The horizontal resolution is 0.22 µm and the vertical resolution is 0.01 µm.
3) Acquisition modes: optical light, non-confocal laser (LSM), and confocal laser (CLSM).
4) A motorized stage that permits moving quickly to the region of interest.
5) The change of the objective lens is executed electrically through PC control.
6) The raster of the laser scan can be reduced up to an additional magnification of 14x without need for changing the lens.

3.2 Procedure

After tooth extraction, a silicone print of the post-extraction site was taken (fluid silicone Aquasil Ultra LV on putty silicone Aquasil Soft Putty, Dentsply®). After that, the silicone print was emptied with plaster (Vel-Mix Stone, ISO type IV, Kerr®) to obtain the study cast. New silicone prints were taken to assess contour changes in the extraction area at one and three months after tooth extraction. The study casts can be observed with CLSM to assess the differences in alveolar socket profiles at one and three months after tooth extraction. Figure 2 shows the images obtained using CLSM.

![Fig. 2 Images obtained with CLSM: a) three-dimensional image and b) bidimensional image.](image_url)

The measurements taken with the CLSM to assess the differences in alveolar socket profiles were:
1) Mesio-distal distance: it measures the mesio-distal distance between the teeth adjacent to the post-extraction site at baseline, one month and three months after extraction to assess possible movements of the teeth towards the edentulous space. Figure 3 shows the mesio-distal distance.
2) Alveolar ridge thickness: it measures the vestibular-lingual width of the alveolar ridge in the post-extraction site at baseline, one month and three months after tooth extraction. Figure 4 shows the alveolar ridge thickness.
3) Vestibular and lingual alveolar ridge height: it measures the distance at which the lingual and vestibular alveolar ridges are placed in relation to the highest point of the gingiva in the mesial part of the extraction site. As the reference point of the gingiva could change one and three months after the tooth extraction, the following measures were used:

a) $H_0$: it measures the distance from the top of the mesial tooth of the extraction site to the highest point of the gingiva in the mesial part of the edentulous space at baseline.

b) $H_1$: it measures the distance from the top of the mesial tooth of the extraction site to the highest point of the gingiva in the mesial part of the edentulous space after one month.

c) $H_3$: it measures the distance from the top of the mesial tooth of the extraction site to the highest point of the gingiva in the mesial part of the edentulous space after three months.

The height at which the ridge was at baseline was determined by measuring the distance between the reference point of the gingiva used in $H_0$ and the highest point of the lingual or vestibular alveolar crest in the different outlines, like as shown in Figure 5.

![Fig. 5](image)

**Fig. 5** a) CLSM image with the transversal sections of the post-extraction area: purple, passing through the top of the mesial tooth of the extraction site; red, passing through the highest point of the gingiva in the mesial part of the edentulous space; yellow, transversal section of the edentulous space, and b) outlines of these sections, where it is possible to obtain the $H_0$ and the vestibular and lingual alveolar ridge height.

To determine the height at one and three months, the distance between the reference point of the gingiva used in $H_1$ and $H_3$ and the highest point of the lingual or vestibular alveolar ridge was measured in the different outlines. Figure 6 shows the schematic illustration of the outlines at baseline and one month following tooth extraction.
In cases where the laser did not reach the bottom of the sample, after taking the mesio-distal distance and the height from the top of the mesial tooth of the extraction site to the highest point of the gingiva in the mesial part of the edentulous space, the teeth were cut down to the gingiva with a Dremel® device to allow the arrival of the laser. Later, microscopic observation was made again. In this way the morphological changes of the edentulous area that occur as a result of the remodelling of the alveolar process after tooth extraction are obtained.

Finally, the acquisition of images with CLSM also allows three-dimensional reconstruction with an image analysis program like the SigmaPlot® software (Systat Software Inc., USA). This program can overlap three-dimensional reconstructions obtained at baseline, one and three months after tooth extraction. The image overlap shows the remodelling pattern of the post-extraction socket. Figure 7 shows the overlap of the three-dimensional reconstruction images. Sectioning the overlap of the three-dimensional reconstruction images, profiles of the edentulous space are obtained at baseline, one and three months after tooth extraction. Therefore, the remodelling pattern of the post-extraction sites can be known.
4. Conclusions

CLSM is an important issue in dentistry studies. CLSM presents different advantages over optical microscopy such as higher horizontal and vertical resolution and three-dimensional reconstruction. CLSM also maintains specimens under constant humid conditions, avoiding artifacts that could occur with SEM drying specimen preparation techniques. Moreover, the resolution of the images obtained with CLSM is similar to those acquired with SEM. Different applications of CLSM to dentistry research have been presented in this chapter.

CLSM is a non-invasive, harmless and valid method for measuring the dimensional changes in post-extraction sockets. This technique allows the visualization of the soft tissues of post-extraction sockets, which can not be collected by CT. The study of post-extraction sites with the methodology presented in this paper allows the assessment of morphological changes after tooth extraction. Differences that occur along the time in the mesio-distal distance, width and height of the alveolar ridge can be measured. This technique with the computer processing of the data allows the three-dimensional reconstruction of the post-extraction changes. Alveolar remodelling pattern can be identified through the overlapping of the three-dimensional images. CLSM has proved to be a powerful tool for the study of the morphological changes of the post-extraction sites.

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