REVIEW

Turk Endod J 2021;6(2):55–60
doi: 10.14744/TEJ.2021.96977

The use of confocal laser scanning microscopy in endodontics: A literature review

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Confocal laser scanning microscopy (CLSM) enables three-dimensional (3D) imaging of tissues and cells marked with fluorescence dyes. Recent technological developments increase the photon efficiency of the CLSM, making it a popular choice for scientific imaging studies. Consequently, CLSM has come to play an essential role in assessing materials used in the field of endodontics as it allows realistic visualization of the endodontic tissues using high-resolution images.

Keywords: CLSM, confocal laser scanning microscope, imaging systems.

Introduction

Confocal laser scanning microscopy (CLSM), which uses an optical microscope first invented by Marvin Minsky in 1955 (1), has rapidly become one of the most commonly used fluorescence microscopic techniques since its launch in the late 1980s, particularly in three-dimensional (3D) studies involving biological cells and tissues. The flexibility of this approach makes it suitable for use in various fields including fast imaging of dynamic processes in living cells, sensitive morphological analysis of tissues, and co-localization of protein expression models (2).

In recent years, the uses of CLSM in endodontic studies have expanded to include observation of the effects of endodontic irrigation solutions on biofilms (3), sealer penetration into the dentin (4), and assessing sealer remnants in the root canal following re-treatment (5). The current review aims to provide general overview about confocal laser scanning microscopes and its uses in various endodontic studies.

CLSM Operation Principles

CLSM consists of a laser beam that functions as a source of light and an electronic system that processes the image produced (Fig. 1). High-resolution optic images are obtained by using extremely thin cross-sections (0.5–1.5 µm), thus eliminating light interference caused by different optical fields across the sample thickness (6). The laser beam passes through a narrow gap called the pinhole, reflects against the sample, and goes back into the microscope to refocus and pass through the pinhole again.

The reflected light is identified with the help of scanning mirrors, and the location of the pinhole can be fixed depending on the sample layer that will be visible through the microscope. This system is called confocal as the returning ray and the image both have a common focal point. CLSM focuses on a single plane, known as optical sectioning, and the points outside this image plane block out any scattered or diffused light, thus generating focused, high-contrast, clear images of relatively thick samples. The

Cite this article as: Yolcu EN, Akın Tartuk G, Kaya S, Eskibağlar M. The use of confocal laser scanning microscopy in endodontics: A literature review. Turk Endod J 2021;6:55-60.
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Submitted: May 04, 2021 Accepted: June 25, 2021

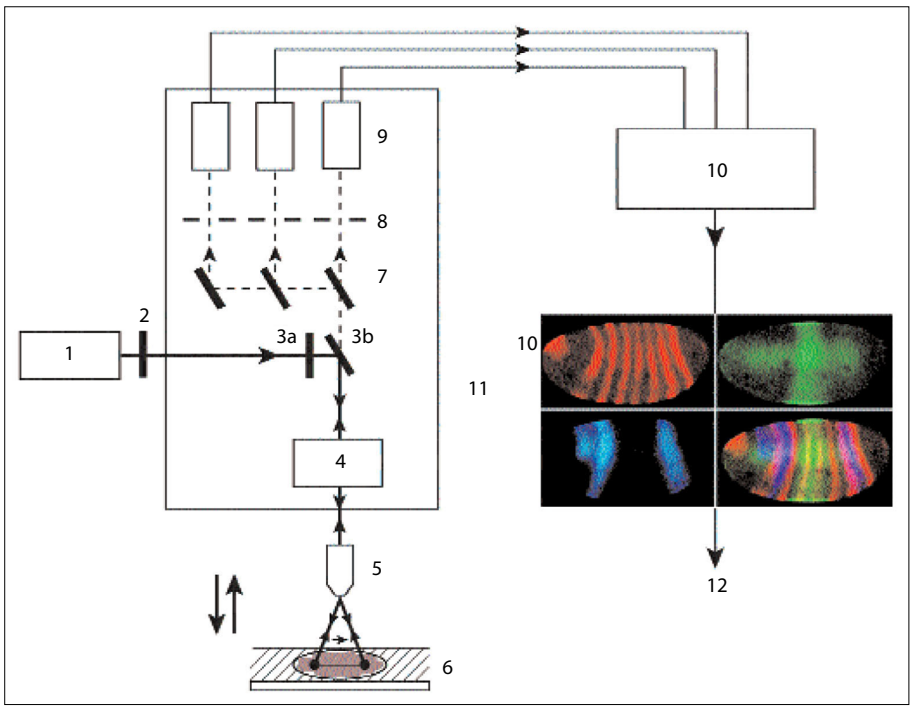

Fig. 1. CLSM's working model: One or more laser light are transferred to (1) a filter wheel, (2) mounted neutral density filters, (3a) excitor and (3b) dichromatic filters, (4) a scanning unit, (5) an objective lens, (6) a sample, (7) emission filters and dichromatic mirrors, small holes (8) placed in front of one or multiple photomultiplier tube (9) (PMTs), (10) a computer and digital control unit, (11) a high-resolution video image, and (12) digital images, high-resolution digital printers, other computers, or the World Wide Web.

images collected from multiple layers of a single sample are then combined digitally using a computer, enabling 3D reconstruction of the complex sample structure (7).

The advantages of CLSM are as follows (8):

1) The samples are kept under constant humidity conditions in CLSM.
2) It allows visualization of the images at higher resolutions.
3) Thin optical sections on different planes can be included.
4) Regions other than the focus plane are not scanned, resulting in high-contrast images.
5) 3D reconstruction with optical sectioning is possible. The sections obtained from different focal planes can be used to create a 3D image of the analyzed sample.
6) The image can be digitalized.

The disadvantages of CLSM are as follows (8):

1) Laser lines or excitation wavelengths occur at extremely narrow bandwidths, and it is costly to generate these rays at ultraviolet wavelengths.
2) The high-intensity laser beam used in CLSM may have damaging effects on live tissues.
3) The high costs limit its usage in dentistry.

CLSM Use in Endodontics

In endodontics, CLSM is used for investigating the penetration depth of irrigation solutions (9–11), medicaments (12), and sealers (13,14) into the dentin. Although these can also be investigated using a scanning electron microscope (SEM), CLSM has become increasingly popular due to its advantages over SEM. Firstly, sample preparation for SEM requires specific steps such as the application of a gold coating which can damage the samples; however, no such specific steps are necessary for CLSM sample preparation, permitting imaging without causing damage to the samples (15). Secondly, interpreting SEM images in studies that evaluate canal sealer penetration into dentinal tubules can be challenging as it is often hard to distinguish between the dentin and the sealer present in the canals. In contrast, CLSM images are easily distinguishable due to the addition of fluorescent dyes to the root canal sealer (16). Thirdly, images from different depths can be combined to create the final image in CLSM (17). In contrast, the magnification of SEM is higher than that of CLSM, thus making imaging and evaluation of the entire surface area challenging. The use of fluorescent materials for improved imaging clarity allows evaluation of larger areas with the smaller magnification of CLSM (18). Additionally, CLSM also

enables imaging of the regions under the smear layer of the dentin surface (19).

Investigating Canal Sealer Penetration Depth

The evaluation of canal sealer penetration using CLSM requires the use of fluorescent Rhodamine B dye to allow differentiation of the sealer material from the dentinal tubules. Rhodamine B produces a powerful visual indication at relatively low concentrations (20), with one study that compared the addition of varying concentrations of the dye to the canal sealer reporting that concentrations higher than 0.1% caused excessive fluorescence in images (15). Rhodamine B has a high solubility structure and is added to dental materials after mixing with a solvent. It can be used with different solvents such as alcohol (21), deionized water (22), distilled water (23), saline solution (24), and aqueous solution (25), although a review of current literature on the use of fluorescent dyes with dental materials showed a lack of consensus on the ideal concentration and solvent type (20). This dye does not cause any changes to the physical properties of canal sealers (20). Cross-sections of the samples are collected to assess sealer penetration, and these samples are then evaluated using CLSM. Failure of sealer penetration into the dentinal tubules may result in the formation of gaps between the canal wall and the sealer, creating potential areas for microleakage from the apical and coronal directions (26).

Chandra et al. (13) evaluated the dentin tubule penetration depth of AH Plus, RealSeal, EndoRez, and RoekoSeal root canal sealers using CLSM and found that RealSeal canal sealer exhibited maximum penetration. Additionally, the researchers stated that the maximum penetration was observed in the coronal part, followed by the middle and apical parts.

Tedesco et al. (27) compared the use of CLSM and SEM images for the evaluation of sealer penetration into the radicular dentin and assessment of the properties of the sealer-dentin adhesion surface. Comparing the two imaging methods for intra-tubular penetration scoring of Endofill and AH Plus root canal sealers showed better imaging outcomes with CLSM. Furthermore, analysis of the adhesive surface formed between the Endofill canal sealer and dentin showed similar results with both imaging methods.

Yamada et al. (28) evaluated and compared SEM and CLSM imaging of the surface profiles of Nd: YAG laser-irradiated enamel and dentin and found that the two methods exhibited different contrasts, with some enamel grooves that were not visible in the SEM images becoming more easily recognizable in the CLSM images due to higher contrast. Comparison of the SEM and CLSM images of the laser-irradiated dentin surface showed that the contouring of the melted globules appeared brighter in the SEM image and black in the CLSM image. Moreover, the sub-surface layer of the dentinal tubules could be observed clearly in the CLSM images but not in the SEM images.

Kuçi et al. (29) used CLSM imaging to assess the tubule penetration of canal sealers in the presence of a smear layer. MTA Fillapex and AH 26 root canal sealers were applied using cold lateral condensation and warm vertical compaction. The researchers found that smear layer removal did not significantly increase the penetration depth of the AH 26 sealer, and application of the MTA Fillapex sealer while cold lateral condensation technique increased penetration depth. Additionally, CLSM imaging showed that MTA Fillapex had better tubule penetration when using the cold lateral condensation technique, while the AH 26 root canal sealer had better tubule penetration with warm vertical compaction.

Uzunoğlu Özyürek et al. (30) preferred using CLSM imaging to evaluate the effects of $Ca(OH)_2$ dressings on the dentinal tubule penetration of AH 26 and BioRoot RCS. The researchers found that the BioRoot RCS canal sealer showed higher penetration depth than AH 26 despite presence of calcium hydroxide residues in the dentinal tubules.

Analyzing Irrigation Effectiveness

CLSM has becoming increasingly popular for the evaluation and comparison of the efficacy of irrigation solutions used in endodontics (31). However, examining the antibacterial activities and effects of irrigation solutions on biofilm using CLSM necessitates the use of various dyes with fluorescent properties (1).

Küçük et al. (32) used CLSM to investigate the effects of using an endo-activator and erbium, chromium: yttrium, scandium, gallium, and garnet (Er, Cr: YSGG) laser-activated irrigation methods on chlorohexidine, QMix, and irritrol penetration. All final irrigants were mixed with 0.01% fluorescent Rhodamine B isothiocyanate to allow visualization within the dentinal tubules using CLSM. The researchers found that QMix exhibited a higher penetration percentage compared to CHX in the apical part, irrespective of the irrigation method used. No statistically significant differences were observed in the middle and apical parts, and sonic irrigation increased the penetration percentage of the irritrol group.

Guerreiro-Tanomaru et al. (3) studied the effects of adding cetrimide to classical irrigation solutions on biofilm using CLSM.

Flach et al. (33) used CLSM to investigate the effects of 2.5% sodium hypochlorite (NaOCl), 2% chlorhexidine

gel, and 2.5% NaOCl + 2% chlorhexidine gel irrigation solutions on *Enterococcus faecalis*, and found that none of the tested irrigation solutions completely eliminated *E. faecalis* from the root canal cavity.

Azim et al. (31) used CLSM to investigate the effects of four different irrigation protocols on bacteria colonized in dentinal tubules. They first autoclaved and prepared the root canals and incubated them with *E. faecalis* for three weeks. The canals were then disinfected by standard needle irrigation, and sonically agitated with EndoActivator, XP Endo finisher, or erbium: yttrium aluminum garnet laser. XP Endo was more effective in disinfecting the main canal space and dentinal tubules up to a depth of 50 µm when compared to the other techniques.

Akcay et al. (34) used CLSM to investigate the effects of Er: YAG-PIPS, Er: YAG-Preciso-type sonic activation, and passive ultrasonic activation on the penetration of the final irrigation solution (2.5 mL of 5% NaOCl) labeled with 2.5 mL of 0.1% fluorescent Rhodamine B isothiocyanate. The researchers concluded that passive ultrasonic irrigation exhibited a higher penetration percentage compared to the sonic activation groups.

Use in Endodontic Microbiology

Bacterial invasion of the dentin in the presence of endodontic infections can be examined using various methods such as SEM, transmission electron microscopy, histological sectioning, and microbiological analyses at different levels of the root canal system. These procedures have their advantages and disadvantages (35). Although microbiological studies can determine the number of colony-forming units of bacteria, it does not provide any information on the spatial scattering of the bacteria inside the dentin (36). Histological examination of sections allows visualization of the distribution of bacteria in the infected dentin, although no information on the viability of the bacteria is available (37). Transmission electron microscopy permits visualization of infected dentinal tubules by providing high-resolution images; however, this technique takes time and requires multiple steps for specimen preparation (38).

CLSM evaluation utilizes thin sections (measuring up to 0.3 µm) of intact biological samples, and is commonly used in combination with vital staining techniques to allow the determination of the viability profile, architecture, and spatial distribution of microbial biofilms (39).

A group of researchers used CLSM imaging of dentin disks infected with *E. faecalis* to examine the interactions between the organism and NaOCl over different durations and at varying temperatures. To allow visualization of *E. faecalis* with CLSM, the samples were stained by first vigorously agitating them with 20 mg/mL calcein-AM in the buffer solution containing 0.25 g dipotassium phosphate and 0.5 g/L sodium chloride. They were followed for 2 hours at 37°C by stained with 3 mL/mL 20 mmol/L propidium iodide for 30 minutes. Calcein-AM, which stains the DNA of target cells, diffuses passively into the cytoplasm and converts it into green fluorescent calcein via native esterases. Upon examining 3D images of the sample cross-sections, the researchers found that the increased contact time with NaOCl was more effective in eliminating *E. faecalis* from the dentinal tubules, although temperature changes did not exert any such effect (40).

Jardine et al. (41) used CLSM to evaluate the effects of NeoMTA Plus, Biodentine, and MTA Angelus on human dentin disks containing biofilm formation. The researchers found that none of the materials had a complete effect on the biofilm, and more than 50% of living bacteria persisted in all groups.

Ma et al. (42) used CLSM as a non-invasive model to assess dentin disinfection efficacy. In that study, they infected the dentinal tubules by centrifuging the *E. faecalis* bacterial suspension. Electron microscopy was used to confirm bacterial existence, and the dentin pieces were then exposed to NaOCl, CHX, distilled water, and QMix. CLSM and viability staining was used to quantitatively analyze the proportions of dead and live bacteria in the dentin. The researchers found no statistically significant differences between the NaOCl, CHX, and QMix groups.

Conclusion

With rapid developments in fast scanning, high-resolution 3D imaging and technology, CLSM has become a commonly preferred imaging system in the field of endodontics. It now forms an indispensable part of biological optic studies and should be adapted to further technological developments to allow continuous use in research.

Authorship Contributions: Concept: S.K.; Design: E.N.Y.; Supervision: G.A.T.; Materials: M.E.; Data: S.K.; Analysis: G.A.T.; Literature search: S.K., G.A.T.; Writing: E.N.Y., G.A.T.; Critical revision: E.N.Y., G.A.T.

Source of Funding: None declared.

Conflict of Interest: None declared.

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