

Article

A Comparison of Two Fluorescent Stains for the Evaluation of Bioceramic Sealer Penetration into the Tubular Dentine Using Confocal Microscopy

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Abstract: This study aimed to compare the percentage of bioceramic sealer penetration and maximum penetration sealer depending on the fluorescent staining method when evaluated with confocal laser scanning microscopy (CLSM). In total, 42 single-rooted premolars were instrumented. Two groups of 20 teeth each were filled using the single cone technique. TotalFill BC Sealer[®] mixed with Rhodamine B was used for the first group and TotalFill BC Sealer[®] mixed with Fluo-3 for the second group. Two teeth made up the control group. All teeth were sectioned at 3 and 6 mm from the apex and were analyzed with CLSM. The canal perimeter where the tubular penetration happened and the maximum penetration of each sample were calculated. There were no significant differences between the two groups in either the apical or medial sections. In terms of maximum penetration, Rhodamine B had greater tubular penetration in the apical sections, and it had similar results to Fluo-3 in the medial sections. TotalFill BC Sealer[®] has a higher tubular penetration in the medial slices than in the apical slices regardless of the fluorescent stain used. Rhodamine B has higher results than Fluo-3 in terms of the percentage of sealer penetration and maximum penetration.

Keywords: bioceramic sealer; confocal laser scanning microscopy; Fluo-3; Rhodamine B; root canal filling



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1. Introduction

Root canal treatment is a complex practice whose success is based on diagnosis and treatment planning, knowledge of the anatomy and configuration of the canal system, a correct debridement, deep disinfection, and a three-dimensional filling of the tooth [1]. Scientific evidence shows that most of the endodontic therapy failures are caused by microorganisms involved in intraradicular and extraradicular infections [2]. Even so, according to several studies, root canal systems cannot be completely disinfected and cleaned [3]; an appropriate pulp canal filling may largely prevent possible filtration [4]. Thus, the purpose of obturation is to provide an impermeable, three-dimensional, and hermetic seal to prevent coronal and apical leakage of microorganisms [4].

Bioceramic sealers have been available for the last thirty years for endodontic use. A great number of these endodontic sealers contain calcium phosphate, which enhances its setting properties and results in a crystalline structure and chemical composition similar to hydroxyapatite [5], improving the bonding of the root dentine to the sealer [5].

TotalFill BC Sealer® is a bioceramic sealer recognized for its physicochemical and biological properties: biocompatibility, antibacterial activity, and bioactivity. This sealer uses the humidity of the tubular dentine for its setting reaction, which extends from 4 h to more than 10 h in a very dry environment [6].

Because of the need to demonstrate the tubular infiltration of the bioceramic sealers, many studies have used laser confocal microscopy (CLSM) to visualize this penetration into the dentinal tubules [7–9]. Thus, CLSM is a useful method for assessing sealer penetration into dentinal tubules [7]. However, to promote an adequate vision, fluorophores associated with endodontic sealers must be used [8]. Fluorophores are stains used to see sealers clearly under CLSM. Most studies have evaluated the penetrability of bioceramic sealers within tubular dentine using CLSM and Rhodamine B as a fluorescent stain [9]. This fluorophore is soluble in water and when diluted it can produce fluorescence [8]. Thus, dentine penetration results could probably be overestimated if Rhodamine B is used, due to the hydrophilic nature of calcium silicate-based sealers [8]. Fluo-3 is another fluorescent stain that could be used for assessing calcium silicate sealers' penetration into the tubular dentine. It is an indicator that significantly increases fluorescence after mixing with calcium [8]. Calcium in calcium silicate-based sealers joins to Fluo-3 and promotes the sealer fluorescence observed in CLSM [10].

Due to the high dissolution of Rhodamine B in water and the behavior of Fluo-3 with calcium ions from bioceramic sealers, it is appropriate to investigate the real behavior of calcium silicate sealers associated with these two fluorophores.

Therefore, the aim of this study was to compare the percentage of sealer penetration and maximum sealer penetration of a bioceramic sealer when associated with two different fluorescent staining methods (Rhodamine B or Fluo-3) through the use of CLSM.

2. Materials and Methods

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE 2021 guidelines) (Nagen-drababu et al. 2021, doi: 10.1111/iej.13542). Additionally, this investigation was accepted by the research committee of the institution where the study was carried out (CIP/213006.50).

2.1. Sample Selection and Endodontic Procedures

The sample size was calculated considering previous research related to tubular penetration.

Forty-two human single-rooted premolars extracted because of suffering periodontal disease were selected with the following exclusion criteria: roots with root caries or not completely formed root, canals with calcific metamorphosis, fissures or fractures, root resorptions, previously treated teeth and canals presenting double curvature, and the presence of more than one canal. Periapical radiographs of each tooth in buccolingual and mesio-distal directions were made to confirm the presence of one canal.

Using an ultrasonic tip, calculus was removed from all the roots and stored for one day in a 2% thymol solution. Subsequently, they were decoronated to achieve a canal size of 12 mm in length. A 08 K-file (Dentsply Maillefer, Ballaigues, Switzerland) was inserted into the canals until it was visualized in the apical foramen. To determine the working length, this length was reduced by 0.5 mm. After the insertion of a 10 K-file (Dentsply Maillefer, Ballaigues, Switzerland), each canal was instrumented with the files of the basic sequence of the Blue Shaper® system (Zarc4endo, Gijón, Spain) up to the working length at 500 rpm (4.0 Ncm). The Eighteenth E-Connect S motor (Town, Xinbei District, Changzhou City, Jiangsu Province, China) was used. Irrigation with 2 mL of 5.25% NaOCl (masterful formulation) between files was performed with a Monojet 27G syringe (Cardinal Health 7000 Cardinal Place Dublin, OH 43017, USA) at 2 mm away from the length where the needle is embedded. Patency of the canals was ensured during all the treatments. Each file was used in 3 canals. To create a closed system, the apical third of the teeth was blocked with Reus wax [11].

Three mL of 5.25% NaOCl and one mL of 17% EDTA were used and both solutions were activated in three 20-s periods renewing the irrigant between them. A final wash was performed with 5.25% NaOCl. This activation was completed with the Ultra X ultrasonic activator (Eighteeth, Jiangsu) at a frequency of 45 kHz using the blue tip (25/02) (Eighteeth, Jiangsu) at one less millimeter than the working length inside the canals.

The canals were dried with 25/02 paper points (Dentsply Maillefer, Ballaigues, Switzerland). Then, using a 25 verifier (Dentsply Maillefer, Ballaigues, Switzerland) an apical calibration was performed. Samples with an interior diameter larger than #25 were discarded and replaced with another tooth. After apical calibration, the teeth were differentiated into two groups depending on the fluorescent staining (Rhodamine B or FLuo-3). Two teeth were used as the control group.

Twenty teeth in the first group were obturated performing the single-cone technique and using TotalFill BC Sealer[®] mixed with the fluorescent stain Rhodamine B (Alquera Ciencia S.L, Cadiz, Spain) at a concentration of 0.1%. In the second group, a further 20 teeth were filled using the single-cone technique and TotalFill BC Sealer[®] mixed with Fluo-3 (Invitrogen, Carlsbad, CA, USA) fluorescent stain at a concentration of 0.1%. In both groups, the single-cone technique was performed with Autofit 06 gutta-percha calibrated to 0.25 mm with a gutta-percha calibrator (Dentsply Maillefer, Ballaigues, Switzerland).

One control tooth was finally irrigated with 1 mL of deionized water in which Rhodamine B was dissolved at a concentration of 0.1%, and the other control tooth was irrigated with 1 mL of deionized water in which Fluo-3 was dissolved at a concentration of 0.1%. Both control teeth were dried briefly to avoid excess moisture. All concentrations were calculated using a microbalance.

All filled canals were sealed with Cavit[®] (3M ESPE AG.Dental Products, Seefeld, Germany). The samples were radiographed to check the obturation and stored at 37 °C and 100% humidity.

2.2. Sample Preparation for CLSM and Images Acquisition

After placing the tooth inside a resin block, each tooth was cut perpendicularly to the longitudinal axis of the root at 3 and 6 mm, with a 0.2 mm precision sectioning saw (Isomet 100 precision saw, Buehler, Lake Bluff, IL, USA) at 100 rpm and under water cooling.

They were adhered to slides on which the sample data were recorded. The surfaces were polished using the Exakt 400 Micro Grinding System (Exakt Aparatebau GMBH, Norderstedt, Germany) using 2500 grit and 4000 grit polishing discs (Figure 1).

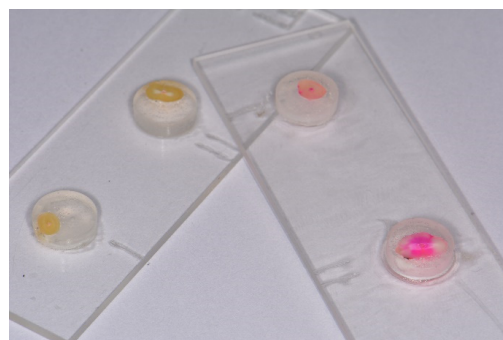


Figure 1. Samples fixed on slides and polished.

The samples were evaluated using scanning laser confocal microscopy (Nikon N-SIM E, Nikon Instruments Inc, Melville, NY 11747-3064, USA). These were scanned at 40× magnification and 10 µm below the surface. The wavelength used for the evaluation of rhodamine was 540/590 nm and 488 nm for the evaluation of Fluo-3.

Digital images were obtained using the fluorescence mode with a resolution of 512 × 512 pixels, resulting in 262144 pixel images. Each pixel was equivalent to 6.31 µm.

2.3. Dentinal Tubule Penetration Analysis

The images were analyzed using Adobe Photoshop software (v.23.5.1) (Adobe Systems, San Jose, CA, USA). A layer named “perimeter” was created where the perimeter of the canal was traced. A layer named “penetration” was created and all areas along the canal walls where the sealer had penetrated the tubules were drawn and measured. The measured distance was divided by the circumference to calculate the percentage of sealer penetration capacity. To describe the maximum penetration depth of the bioceramic sealer, the point of maximum longitudinal penetration was drawn [12,13]. Each measurement was performed twice by two different examiners to confirm their agreement.

2.4. Statistical Analysis

The intraclass correlation coefficient (ICC) was estimated as a measure of inter-examiner reproducibility. For the purposes of subsequent statistical analyses, the average of the measurements obtained from both examiners was used.

Regarding comparing the results of each staining with its control, the 95% CI was estimated for the median of each group and the inclusion of the control in the same group was assessed. The Wilcoxon test was used to evaluate whether there were differences in the results between the apical and medial sections of each group. Finally, a Brunner–Langer model for the related data was used to evaluate and compare the results of the slices between the two groups. The ATS statistic was used to conclude the main and interaction effects.

The significance level used in the analyses was 5% ($\alpha = 0.05$).

All these stages are summarized in Figure 2.

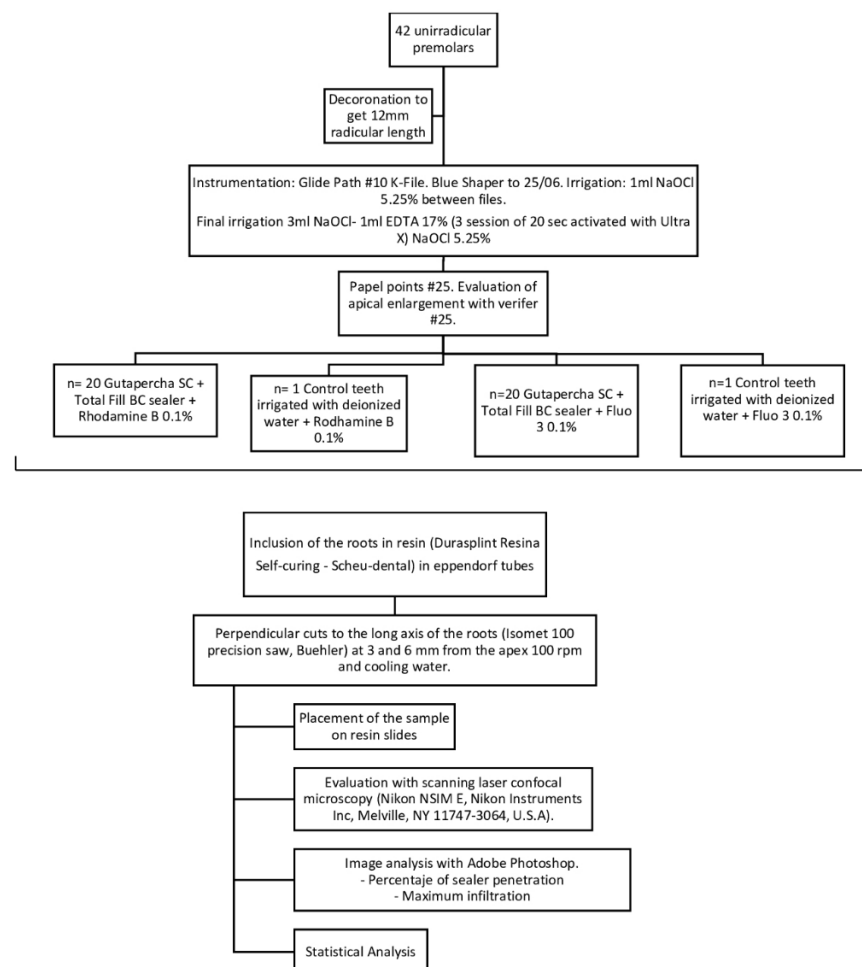


Figure 2. Flow chart of the methods employed.

3. Results

The ICC values around 0.90 allow a high level of agreement between the examiners. Regarding the percentage of sealer penetration in the apical sections, there was no statistical evidence according to the type of staining ($p = 0.141$). However, both Fluo-3 and Rhodamine B had a significantly higher percentage compared with their respective control group ($p < 0.01$) (Figure 3).

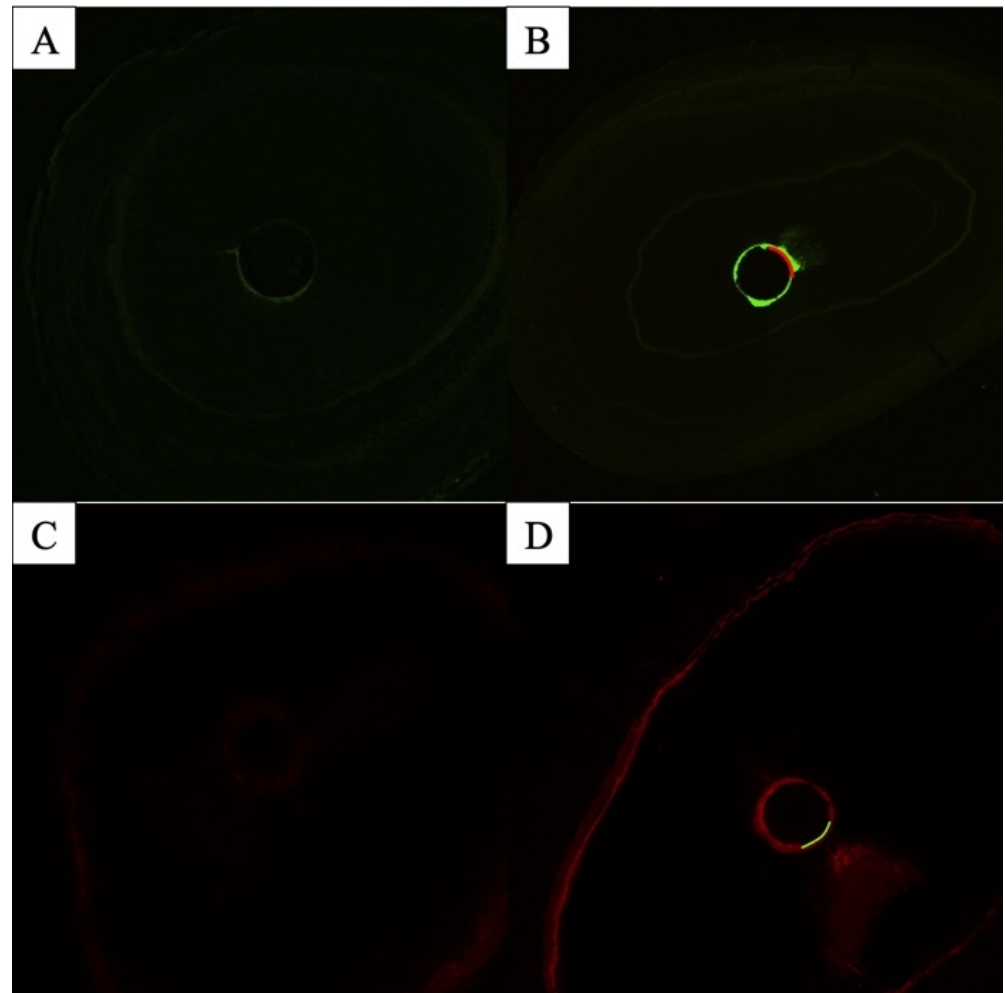


Figure 3. (A) An image of the apical slice of the Fluo-3 control group. (B) An image of an apical slice of the Fluo-3 group. (C) An image of the apical slice of the Rhodamine B control group. (D) An image of an apical slice of the Rhodamine B group. The traced lines (red in image (B) and green in image (D)) show the wall of the canal where the infiltration has happened.

There was no statistical evidence ($p = 0.396$) in the percentage of sealer penetration according to the type of staining in the medial slices (Figure 4).

In these medial slices, the Fluo-3 group had significantly lower tubular percentages than its control group ($p < 0.05$), and the Rhodamine B group had a similar sealer penetration to its control group ($p > 0.05$).

Comparing the apical and medial slices, the percentages of sealer penetration in the medial slices was significantly higher than that in the apical slices in both groups; in the Fluo-3 group $p < 0.001$, and in the Rhodamine group $p = 0.042$, although it had a lower level of reliability (Figure 5).

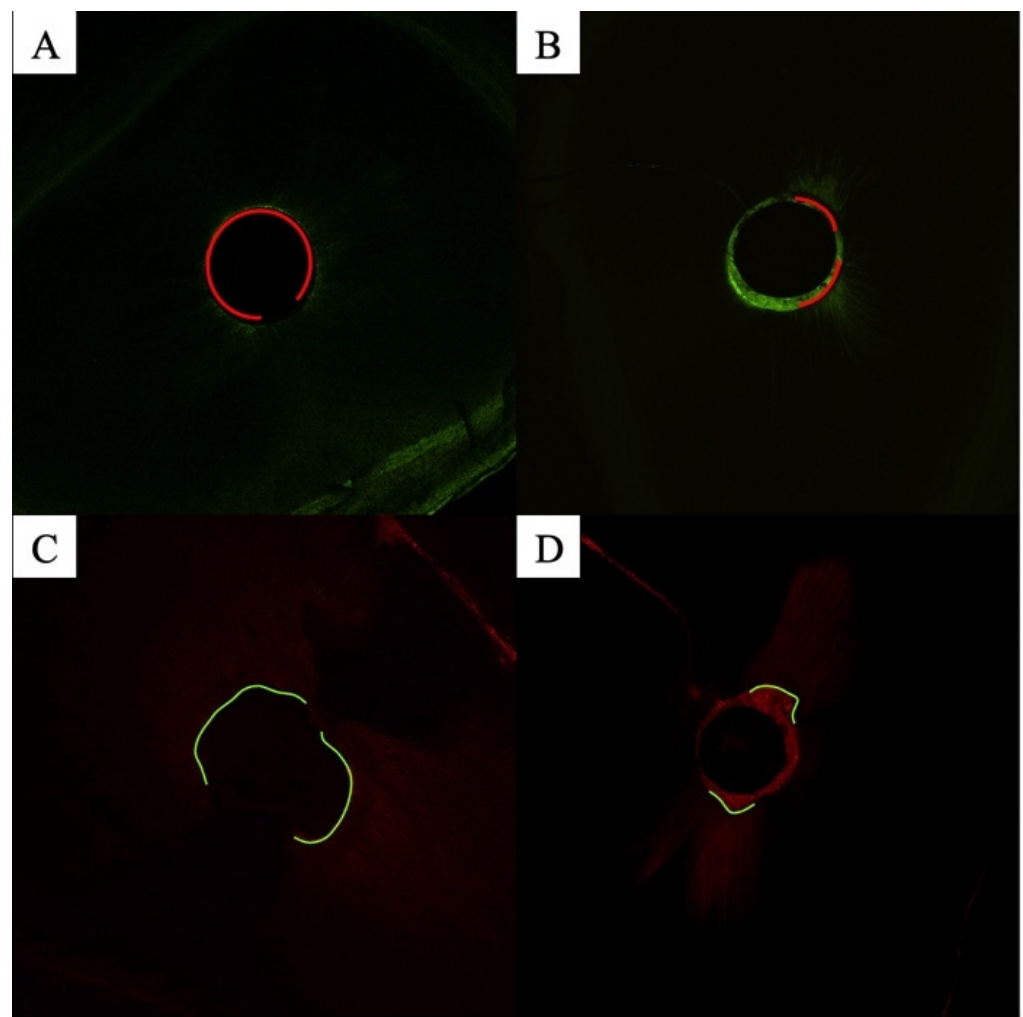


Figure 4. (A) A medial slice image of the Fluo-3 control group. (B) A medial slice image of the Fluo-3 group. (C) A medial slice image of the control group Rhodamine B. (D) A medial slice image of the group Rhodamine B. The traced lines (red in images (A,B) and green in images (C,D)) show the wall of the canal where the infiltration has happened.

Regarding maximum penetration, in the apical slices, Rhodamine-B implied a significantly higher penetration than Fluo-3 ($p = 0.026$) in addition to having significant differences in maximum penetration with its control tooth ($p < 0.01$), as the maximum penetration of the control tooth is significantly lower ($p < 0.01$). The same occurred with Fluo-3, whose maximum penetration was also significantly higher than its control ($p < 0.01$). There were no significant differences in the maximum penetration in the medial slices according to the type of fluorescent staining ($p = 0.181$). Fluo-3 had a significantly higher maximum penetration than its control group ($p < 0.05$), and Rhodamine B obtained similar results to its control ($p > 0.05$). In the Fluo-3 group, the filtration in medial slices was significantly superior to that in apical slices ($p < 0.001$), and in the Rhodamine group the same result occurred ($p = 0.042$), although with much lower level of reliability. Regarding the maximum penetration according to the fluorescent stain used, there were differences between the two groups ($p = 0.008$). With Fluo-3, there were fewer penetrations and these differences were similar in magnitude in both types of slice ($p = 0.230$) (Figure 6).

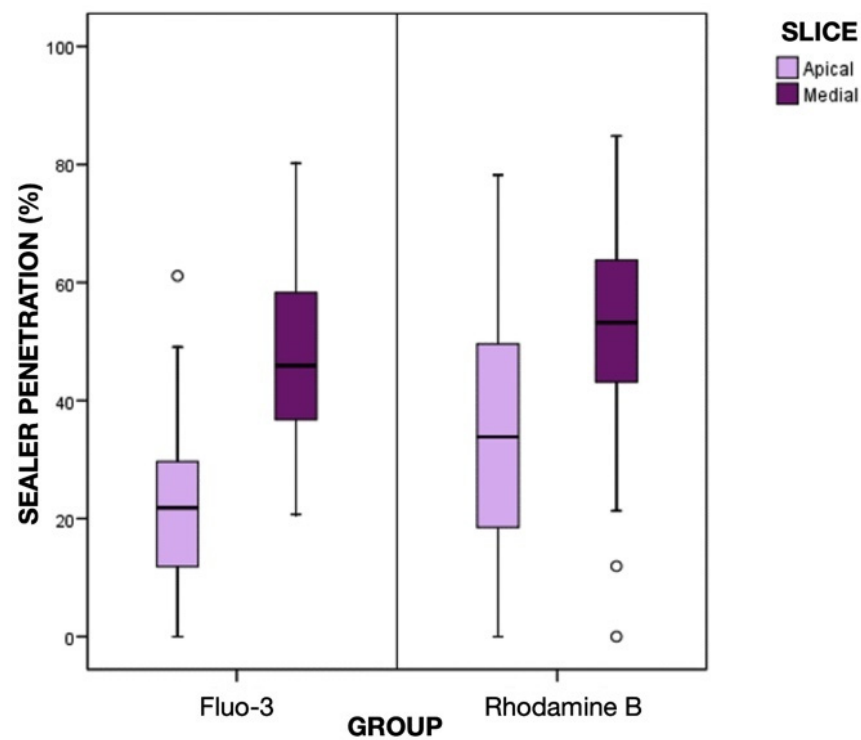


Figure 5. The percentage of sealer penetration according to the apical or medial slice in each sample. The percentages of sealer penetration in medial slices were higher than those in apical slices in both groups.

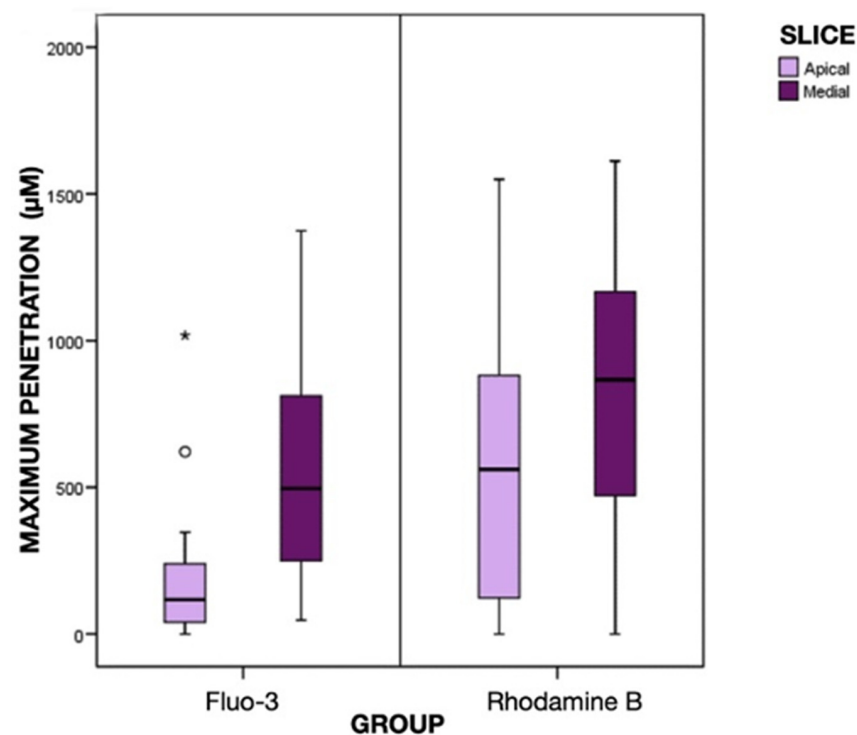


Figure 6. Maximum penetration according to the apical or medial slice in each sample. In the Fluo-3 group, the infiltration in the medial slice was significantly superior to that in the apical slice ($p < 0.001$), and in the Rhodamine group the same result occurred ($p = 0.042$), although with much lower level of reliability. * its related to an apical slice with an extreme value of maximum penetration within the sample.

The interquartile range (IQR) is a way of calculating the dispersion of the samples in each group. It was observed that, in terms of the percentage of sealer penetration, both at the apical and medial levels, the IQRs of Rhodamine B (31.1 and 24.5, respectively) were higher than the IQRs of Fluo-3 (17.8 and 21.5, respectively). Similar data were obtained for the maximum penetration data at the apical and medial levels. Rhodamine B had higher IQR values (757.6 and 780.4, respectively) than the Fluo-3 group (199.8 and 694.1, respectively) (Table 1).

Table 1. Interquartile range of each group.

		Interquartile Range (IQR)	
Percentage of tubular penetration		Fluo-3	Rhodamine B
	Apical	17.8	31.1
	Medial	21.5	24.5
Maximum penetration		Fluo-3	Rhodamine B
	Apical	199.8	757.6
	Medial	694.1	780.4

4. Discussion

TotalFill BC Sealer[®] is one of the new calcium silicate-based sealers that has appeared on the European market. In Canada it is called iRoot BC Sealer, and in the USA, EndoSequence BC Sealer [14]. This bioceramic sealer has been reported by several studies because of its favorable properties as a root canal filling material, including good sealing ability [15], biocompatibility [16], antibacterial activity [15], and dentine adhesive properties [17]. It has proven to have higher tubular penetration data compared to other bioceramic sealers [18], and even in comparison with the resin-based sealer AH Plus [19,20]. In the present study, TotalFill BC Sealer[®] was evaluated, and regardless of the fluorescent staining method evaluated, TotalFill BC Sealer[®] demonstrated, in most of the images acquired with CLSM, that it can infiltrate the dentinal tubules.

One of the performance measures of endodontic sealers is its ability to penetrate the dentinal tubules. According to De-Deus et al. [21], there are no correlations between sealer penetration and the sealing of filling materials. Nevertheless, it has been shown that the penetration of sealers into the dentinal tubule forms a physical barrier [22] that improves the retention of the filling material [23], and residual microorganisms are encapsulated within the canal [24]. To evaluate this, many authors have used Rhodamine B as a marker in their methodology to visualize the sealer inside the tubules [19,20]. Rhodamine B has a strong affinity for humidity and less affinity for the calcium in the sealer composition [8]. As a result, it can separate from its mixture with the sealer, and emit sealer-independent fluorescence, showing a deeper penetration into the dentine tubules [8]. Thus, we found it necessary to check the predictability of Rhodamine B and ensure that, in the present study, the method of evaluating tubular infiltration with this stain is the most appropriate. Fluo-3 is a more appropriate fluorescent stain for tracking calcium ions under CLSM because in the presence of calcium ions, it increases the intensity of its fluorescence [25]. However, Fluo-3 cannot measure the number of calcium ions in the tooth structure [8]. Consequently, the fluorescence produced in CSLM images acquired in this study may be related absolutely to the sealer. With its unique property of generating light in response to the specific wave range of the argon laser (488 nm), Fluo-3 can be used to detect the presence of calcium silicate-based sealers under CLSM [26]. In this study, the results indicated that, in terms of maximum penetration and the percentage of sealer penetration (%), Rhodamine B had higher values throughout, demonstrating significant differences with respect to Fluo-3 when evaluating the maximum penetration at the apical level. Apart from this, it is important to emphasize that the interquartile range (IQR) was always higher in the Rhodamine B group, particularly at these apical levels. This shows the presence of greatly

variable and different data in the Rhodamine B group compared to the Fluo-3 group, where all data were similar (Table 1). Thus, it could be observed that Fluo-3 presents a better performance and a lower variability, showing more homogeneous and more reliable data. In another study that used the calcium-related marker Fluo-3 to investigate the penetration depth of bioceramic sealers, it has been shown that the sealer penetration is lower compared to the values that can be expected from studies using Rhodamine B [27,28], which also corroborates the findings of the present study. Few studies describe the need to introduce Fluo-3 instead of Rhodamine B for the evaluation of tubular penetration. Studies such as those by Jeong et al. [8] and Donnermeyer et al. [29] detailed that the use of Rhodamine B for the assessment of tubular penetration is not the most appropriate technique to visualize this penetration, due to the high affinity of Rhodamine B to humidity, causing the obtainment of results that appear to be higher under CLSM. It is important to indicate the fact that Fluo-3 is an indicator with an unquestionably higher price than Rhodamine B and this increases the financial plan of the studies to a notorious degree. Even so, in the present study, we can observe that the use of Fluo-3 for the evaluation of the tubular penetration of bioceramic sealers should be the best choice to avoid variations that may alter the results.

For the study sample, single-rooted premolars with a single canal were chosen. The ignorance of the age of teeth used and their function in the oral cavity, could have an influence on the tubular obliteration. Thus, an explanation can be given for the existence of extreme cases in the studied groups. Therefore, researchers should consider investigating new methods for standardizing the type of dentin in subsequent studies.

It should be noted that the illumination power of the laser light source in the confocal microscope significantly increases the image intensity [30]. An interesting finding in this study was the statistically lower percentage of sealer penetration of Fluo-3 in medial slices compared to its control tooth at this level ($p < 0.05$). If Fluo-3 increases its fluorescence in the presence of calcium ions dissociated from the sealer, it was surprising that the tubular penetration ability was statistically higher in the control tooth. The explanation given for this finding was the presence of a higher permeability of the dentinal tubules and a possible overexposure of the sample. Furthermore, it should be noted that more teeth should have been used in the control group to allow for more statistical variability.

The method of analyzing the tubular penetration capacity and the maximum penetration of the sealer into the dentinal tubules was based on the methodology of Gharib et al. [12] and Bolles et al. [13], among others. This method is a way of analyzing penetration that has a certain subjectivity [31]. The main problem with any image-based methodology is its extrapolation to numerical values. Even so, two trained researchers carried out the measurements to try to minimize this fact. Using the intraclass correlation coefficient (ICC), agreement values of around 0.90 were obtained, concluding a high level of affinity between the examiners.

Further investigations on the tubular penetration of sealers and methodological approaches by the direct detection of sealers in tubules below the surface of a specimen are needed. Furthermore, it seems likely that the penetration of the sealer into the dentine tubule does not contribute relevantly to canal sealing [21]. Likewise, penetration into the tubule must be considered in combination with other factors, such as sealability and bond strength.

Following the conditions of this study and independent of the fluorescent stain used, there is less tubular penetration in the apical sections than in the medial sections of the dental root when a calcium silicate-based sealer is used.

Rhodamine B achieves a higher tubular penetration than the fluorescent stain Fluo-3, but there are only significant differences between groups when comparing the maximum penetration at the apical level.

A better performance and less statistical variability was observed in the Fluo-3 group; thus, we conclude that it has more reliable results. The best method to evaluate tubular penetration would be using the Fluo-3 fluorescent stain.

Further studies about the tubular penetration of endodontic sealers are needed.

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Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

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