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Creation of well-balanced experimental groups for comparative endodontic laboratorial studies: a new proposal based on micro-CT and *in silico* methods

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Keywords: anatomy, mandibular incisor, micro-CT, root canal, sampling method.

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Abstract

Aim To introduce a new method to select anatomically matched teeth using micro-computed tomographic (micro-CT) technology.

Methodology Single-rooted mandibular incisors with a single root canal (n = 60) were selected and distributed into three experimental groups according to the method used for matching 10 pairs of teeth in each group. In group 1, the pairs of mandibular incisors were randomly selected from a pool of teeth. In group 2, teeth were paired based on the measurement of canal width 5 mm from the root apex using radiographs taken from buccolingual and mesiodistal directions. In group 3, teeth were scanned (pixel size of 14.25 μm) and pair-matched based on the anatomical aspects of the root canal, named aspect ratio (AR), volume and three-dimensional canal geometry. After allocating the specimens into groups 1 and 2, the teeth were scanned and the canal morphology evaluated as in group 3. A bivariate Pearson's regression analysis was performed correlating the individual AR values of each pair and the correlation coefficient was used to estimate the strength of the pair-matching process. One-way ANOVA *post hoc* Tukey tests were applied for pair-wise comparisons at a significance level of 5%.

Results The micro-CT method showed 100% of the samples having strong (80%) or very strong (20%) correlations with respect to AR values. Analysis of the radiographic method revealed strong correlation in two pairs (20%), but most of the samples had weak (30%) or neglectable (30%) correlation coefficients. The randomization method resulted in 3 pairs (30%) with very strong correlations, while 50% had weak or neglectable rates. A significant difference in correlation coefficients was observed in the micro-CT method compared to the other groups ($p < 0.05$), whilst no difference was detected between radiographic and randomized methods ($p > 0.05$). Eta squared (η^2) calculations demonstrated a very high effect size in the micro-CT group for selecting pairs (0.99), and lower effect sizes in the radiographic (0.67) and randomized (0.66) groups.

Conclusions Micro-CT method was able to provide better control of the confounding effect that anatomical variances in tooth morphology may have on the results in experiments with matched-pair design.

Keywords: anatomy, mandibular incisor, micro-CT, root canal, sampling method.

Introduction

Experimental bench tests have often been used to screen and rank the quality of a plethora of materials and techniques related to root canal shaping, irrigation, disinfection and filling procedures (Buck *et al.* 1999, Eldeniz & Ørstavik 2009, Çapar *et al.* 2014, Passalidou *et al.* 2018). However, results and conclusions from some of these comparative studies may not be reliable because of a general lack of anatomical matching of teeth prior to experimentation (De-Deus 2012). This lack of standardization reveals an inconsistent and poor internal validity of these studies, which is in line with the statement of Babb and co-authors (2009): "*Although testing designs that use natural canal spaces have pragmatic appeals to clinicians, they have severe limitations from a materials science perspective.*" In fact, the difficulty of creating a reliable baseline using extracted natural teeth is a consequence of the intricate anatomy of the root canal system, which is a decisive confounding factor.

Overall, experimental groups in comparative studies have been created by selecting randomized either single- or multi-rooted teeth with limited sample size (Topçuoğlu *et al.* 2016, Silva *et al.* 2017, Pedullà *et al.* 2019). In practice this means a very poor standardization, unable to ensure experimental comparability as it may yield experimental groups with large variations in the baseline of the substrate (Smith & Steiman 1994, També *et al.* 2014). Consequently, these studies may have been conducted under dissimilar experimental conditions and the results may reveal the effect of the root canal anatomy rather than the variable of interest, i.e. materials, techniques, and/or instruments under comparison (De-Deus 2012). To overcome this problem and optimize the experimental design, anatomical matching of root canal morphology should be regarded as an underlying experimental first step of any comparative *ex vivo* study in endodontics. This will provide a consistent baseline and an improvement of overall internal validity of the study. Therefore, several studies attempted to overcome the anatomical factor using the visual examination of radiographs taken in different angulations (Yared & Bou Dagher 1994, Bürklein & Schäfer 2012) by allocating the teeth into experimental groups according to the root canal width measured at some distance from the root apex (Ruckman *et al.* 2013). However, the overall quality of this methodological approach is not evidence-based. One is led to speculate that this is indeed far from ideal, considering the well-known variations of canal shape throughout the root (Versiani *et al.* 2012, 2016a). Another approach suggests the use of contralateral teeth (Johnsen *et al.* 2017), which have been shown to comprise similar anatomy (Zehnder *et al.* 2006, Mitchell *et al.* 2011, Iriboz *et al.* 2015, Viapiana *et al.* 2016, Guimaraes *et al.* 2017). Through the use of micro-computed tomographic (micro-CT) imaging technology, it was demonstrated that contralateral premolars do indeed exhibit a high degree of matching symmetry and a valid and reliable computer simulation (*in silico*) method was developed for matching contralateral premolars in experimental endodontic comparative studies (Johnsen *et al.* 2016, 2017, 2018). These findings, however, did not preclude the use of other tooth types, but suggested that it would be conceivable to match teeth from different individuals if they are within a certain range of morphological similarity (Johnsen *et al.* 2016). The range, or lowest acceptable similarity coefficient, would certainly rely on further validation, and has yet to be determined. Thus, this type of database repository of non-contralateral pulp spaces would open up for extremely selective, as well as, time and cost-efficient studies.

The purpose of the present study was to introduce a novel methodology for matching non-contralateral mandibular incisors into experimental groups based on their internal morphology through the use of micro-CT imaging technology. This proposal aims to improve the internal validity of comparative studies in endodontics by the creation of anatomically well-balanced experimental groups when compared to conventional methods based on randomization or radiographic examination. To confirm the anatomical similarity of the teeth, data obtained were scrutinized with particular emphasis on the aspect ratio of the canal along the entire root length. This parameter can be considered as a token of morphological similarity between different teeth. Additionally, advantages and limitations of this new proposal were also carefully addressed. The null hypothesis tested was that there would be no difference in the correlation coefficients amongst the three tested methods.

Materials and methods

Sample size calculation

Based on the results of Versiani *et al.* (2013a), it has been estimated an effect size of 0.7 for the selection method to yield adequately anatomically paired samples using micro-CT technology. This value was input into a *t*-test family, correlation bi-serial method at G*Power for Mac 3.1

(Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany), together with an alpha-type error of 0.5 and beta power of 95%. The software indicated a number of 16 samples (8 pairs) per group to observe significant effect of selection criteria using micro-CT over conventional methods based on randomization or radiographic examination.

Sample selection and groups

This study was approved by the local institutional ethics committee (protocol 87450517.5.0000.5243). A total of 1,708 single-rooted mandibular incisors that have been stored for the last 4 years in a tooth bank of our department were available for this experiment. Inclusion criteria comprised only teeth showing no root fracture, calcification, caries, resorption or incomplete root formation. From this set of teeth, three experimental groups were created according to the following methods:

Group 1 – Randomized method (n=20). From a pool of the first 76 mandibular incisors randomly gathered from the original sample (n=1,708), it was possible to collect 20 teeth having 20 ± 1 mm in length (from the incisal edge to the anatomic apex), and randomly distributed them into 2 subgroups (n = 10) with the aid of a computer algorithm (http://www.random.org).

Group 2 – Radiographic method (n=20). From additional 100 mandibular incisors randomly gathered from the original sample (n=1,708), it was possible to select 10 pairs of teeth having similar canal width measured 5 mm from the anatomic apex (FIJI/ImageJ software v.1.51n; Fiji, Madison, WI, USA) by using digital radiographic projections (Schick CDR digital radiographic system; Dentsply Sirona, Charlotte, NC, USA) taken from both buccolingual and mesiodistal directions of each specimen.

Group 3 – Micro-CT method (n = 20). Two-hundred and fifty-one mandibular incisors (n=251) randomly gathered from the original sample (n = 1,708) were necessary for creating of 2 anatomically pair-matched groups (n=10) based on a micro-CT approach. Thus, 251 specimens were scanned

(SkyScan 1173; Bruker-microCT, Kontich, Belgium; 70 kV, 114 mA, pixel size of 14.25 µm, 360° rotation with a rotation step of 0.5°, frame average of 5, using a 1.0-mm-thick aluminium filter) and reconstructed into axial cross-sections (NRecon v.1.7.16 software; Bruker microCT) with individualized parameters for ring artifact correction (3 to 4), contrast limits (0 to 0.05) and beam hardening correction (30% to 45%), resulting in 700-900 grayscale cross-section images per tooth from the cemento-enamel junction to the apex. Then, cross-sectional images were segmented to identify the root canal configuration (Figure 1a) using an automatic routine in FIJI/ImageJ software (Fiji v.1.51n; Fiji). Briefly, non-local means filter (Buades *et al.* 2011) was used to reduce noise while preserving object edges (Figure 1b), followed by applying the Otsu-based algorithm (Otsu 1979) for binarization (Figure 1c). In order to segment the root canal, the 3 stages of 2D "fill holes" were added to the automatic routine after suitable reslicing of the volume to reveal the 3 orthogonal planes (xy, xz, yz). After that, small residual pixels were automatically removed with the 'Keep Largest Region' tool implemented in the MorphoLibJ plugin (Legland & Arganda-Carreras 2014), which allows the identification of the largest connected component, removing the disconnected ones (Figure 1d). From this point, only specimens with a single root canal (Vertucci's type I canal configuration) were used. The volume of interest (VOI) was then established from the cemento-enamel junction to the apex for measuring the aspect ratio and volume parameters of root canal. The aspect ratio (AR) is defined as the ratio of the major to the minor diameter and was calculated at each cross-section from the apposition of an ellipse that best fits the root canal using the Shape Descriptors plugin of FIJI/ImageJ software (Fiji v.1.51n; Fiji) (Figure 1e). The results of AR acquired in all slices were plotted into a graph (Figure 1f) to describe the variations of 2D geometry of the canal throughout the root. Canals with AR close to 1 present a rounded shape, while AR values higher than 3 indicate oval or elongated canal form. Volume (in mm^3) was calculated as the volume of the binarized canal within the VOI using the Object Counter tool (FIJI/ImageJ software). Three-dimensional (3D) models of the root and root canal of each specimen were also created using the CTAn v.1.18.8 software (Bruker microCT) and qualitatively evaluated from both buccal and proximal views with CTVol v.3.3 software (Bruker microCT) (Figure 1g). After that, the allocation of samples into anatomical pairs was performed. Firstly, subgroups of teeth were created according to the root canal volume with a maximum variation range of 2 mm^3. It was determined with the aid of a proper statistical test (Cronbach's alpha test) that indicated a very high homogeneity of the data (0.968) when samples were categorized within this maximum volume variation. Further, the 2D geometry of the entire root canal, represented by the AR graphical curve (Figure 1f), was compared. Teeth categorized within the same canal volume range and showing similar graphical curves, were re-grouped. Finally, the 3D morphological aspect of the root canals (Figure 1g) in these groups was examined and the specimens allocated into 2 groups of anatomical pair-matched teeth (n = 20) based on similar volume, AR plots and 3D renderings of the root canals. Two experienced operators independently double-checked these parameters before sample distribution.

After allocating all of the specimens into the 3 experimental groups, the teeth selected in groups 1 and 2 were also scanned and reconstructed with the SkyScan 1173 micro-CT device (Bruker-microCT) following the same parameters used in group 3. Then, AR values in each cross-section, as well as, the volume and 3D configuration of the root canals were obtained from the specimens of groups 1 and 2 and used for comparison. A detailed flowchart of the methodology is shown in Figure 2.

Statistical analysis

A bivariate Pearson's regression analysis was performed correlating the individual AR values of each pair. The correlation coefficient obtained for each pair was used to estimate the strength of the pair-matching alongside the root lengths following the general rule of correlations strength and categorized as very strong (0.9 to 1.0), strong (0.7 to 0.9), moderate (0.5 to 0.7), weak (0.3 to 0.5) or negligible (0 to 0.3) (Cohen 1988). Then, the correlation coefficients were compared amongst the groups to verify the similarity of their strength regarding the methods used to form pair-matched samples. Because a bell-shaped distribution was observed for the correlation coefficients, a one-way ANOVA procedure was performed followed by a Tukey HSD test for pair-wise comparisons. Additionally, the effect size of each method was calculated using the eta squared (η^2). All statistical analyses were performed using the Statistical Package for Social Sciences software (SPSS v.24; SPSS Inc., Chicago, IL, USA) with a cut-off level for significance adopted at 5%.

Results

Table 1 shows the correlation coefficients of each pair-matched tooth using the three methods of sampling. The micro-CT method (Group 3) showed 100% of the samples rating as strong (80%) or very strong (20%) correlations regarding AR values. Analysis of the radiographic method (Group 2) revealed strong correlation in 2 pairs (20%), but most of the samples had weak (30%) or neglectable (30%) correlation coefficients. Using the randomization method (Group 1), only 1 pair (10%) was rated at very strong correlation and 2 pairs (20%) at strong correlation, while 50% reached weak or neglectable rates. One-way ANOVA *post hoc* Tukey HSD tests found a significant difference in correlation coefficients reached by the micro-CT method compared to the other groups ($p = 0.000$), whilst no difference was detected between the correlations' coefficients of the radiographic and randomized pairs ($p > 0.05$). Eta squared (η^2) calculations demonstrated a very high effect size in the micro-CT group for selecting pairs (0.99), and lower effect sizes in the radiographic (0.67) and randomized (0.66) methods. Figures 3, 4 and 5 illustrates the results obtained from representative pair-matched samples in each group.

Discussion

The major challenge in creating well-balanced experimental groups for comparative endodontic studies is the variations in the intricate anatomy that may exist in a randomized group of teeth. Therefore, one important goal of *ex vivo* laboratory experiments must be to create a feasible method able to overcome the inherent internal variation in natural human teeth (Versiani *et al.* 2013a). Prior to the development of the present proposal, a literature search was conducted to identify the most commonly methods used to create pair-matched teeth samples in experimental studies in endodontology. Basically, these methods aimed to allocate samples into experimental groups according to their anatomical characteristics. It was observed that some studies adopted one point of the root, usually 5 mm from the anatomical apex, and measure the width of the root canal in both buccal and proximal directions, following the methodology of Wu *et al.* (2000), to determine its shape (Tinoco *et al.* 2014, Teixeira *et al.* 2015, Lee *et al.* 2019). In other studies, specimens are allocated from the same group of teeth by randomization (Topçuoğlu *et al.* 2016, Silva *et al.* 2017, Pedullà *et al.* 2019), while some of them adopted a combination of radiographic and randomized methods to form pairs (Ruckman *et al.* 2013, Bernardes *et al.* 2016). Recently, a few studies started to use specific anatomical parameters identified by means of a micro-CT scanning to pair-match samples (Versiani *et al.* 2013b, Johnsen *et al.* 2016, Versiani *et al.* 2016b, Johnsen *et al.* 2017, 2018). In fact, the call for the exploration of a suitable scientific screening and matching protocol for use in endodontics comparative studies was recently raised by Xu *et al.* (2016) in their timely and appropriate proposal to use contralateral premolars for providing baseline consistency. Contrariwise to Johnsen *et al.* (2017), they found relatively few pairs of contralateral premolars with anatomical symmetry. Nevertheless, in concurrence with Johnsen *et al.* (2017), they found that contralateral teeth did have better symmetry than non-paired teeth. Future research should explore if the similarity of screened and matched non-paired teeth through the present proposal of using micro-CT for pair-matching is comparable with contralateral premolars. However, the availability of contralateral premolar teeth with mature apices extracted from younger patients undergoing orthodontic treatment may be limited.

The present study was based on a large human *ex vivo* material of 1,708 mandibular incisors. The teeth underwent a strict selection process to create two anatomically pair-matched groups based on micro-CT and radiographic methods and one group allocated by randomization, with each group consisting of 10 pairs of mandibular incisors. The strict selection method from a large repository of teeth along with proper *a priori* sample size calculation allowed for the possibility to demonstrate which group had the best well-balanced baseline experimental pairs by statistically assessing how strong is the similarity between the internal root canal shape between the pairs, based on the cross-sectional AR values correlations.

The ~~weak and neglectable~~low correlation coefficients obtained from both radiographic and randomization methods in the present study ~~are disconcerting. So, the null hypothesis was rejected. In summary,~~ demonstrate that randomization and radiographic matching methods were unable to overcome the inherent biological variance in root canal anatomy. ~~So~~Thus, the null hypothesis was rejected. These results clearly demonstrated how endodontic comparative studies using unsophisticated screening and matching require an increased sample size to show real and statistically significant differences. Actually, an increase in sample size will likely lead to more precision as individual differences will matter less, but it may reach a point where the effect over precision is meaningless (Souza 2014). It must be pointed out that ethical and economic considerations are also important incentives for not having sample sizes larger than necessary. Therefore, matched and well-balanced groups can provide smaller sample sizes with sufficient power to give reliable results. In fact, the effect that baseline sample matching has on the reduction of sample size has been previously demonstrated with remarkable results in bone research (Banse *et al.* 1996, Barker *et al.* 2005).

The present methodology opens up for the future use of 3D matching and object retrieval methods (Hilaga *et al.* 2001, Osada *et al.* 2001, Tangelder & Veltkamp 2008) with deep learning or artificial neural network capabilities (Hilaga *et al.* 2001, Ekert *et al.* 2019, Krois *et al.* 2019). Such capabilities incorporated into a user-friendly and semi-automated interface would allow for rapid *in silico* selection of teeth with desired root canal morphology, such as oval-shaped canals, and then collect the samples physically from a biobank of teeth available for a multitude of different endodontic comparative experiments with high internal validity. The novel micro-CT method presented herein efficaciously removes the confounding effect that anatomical variances in root canal morphology may have on the results in pair-match experimental designs. This will have unequivocal implications for sample distribution in experimental groups in order to improve the design of comparative studies in endodontics.

Conclusion

Micro-CT method was able to provide better control of the confounding effect that anatomical variances in tooth morphology may have on the results in experiments with pair-matched design.

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Figure legends

Figure 1. Micro-CT processing method used to select pair-matched mandibular incisors applied to representative samples having flattened (sample 1) and round-shaped (sample 2) root canals. (a) The cross-sectional images were submitted to (b) non-local means filter to reduce noise and (c) segmented. Then, (d) the canals were binarized and (e) the aspect ratio calculated and (f) plotted into graphs. (g) 3D models of the root and root canal of each specimen were also created and qualitatively evaluated in both buccal and proximal directions.

Figure 2. Flowchart of the methodology.

Figure 3. (a) Graph of correlation coefficients, (b) 3D rendering and (c) aspect ratio graph of two samples pair-matched based on micro-CT method.

Figure 4. (a) Graph of correlation coefficients, (b) 3D rendering and (c) aspect ratio graph of two samples pair-matched by the radiographic method.

Figure 5. (a) Graph of correlation coefficients, (b) 3D rendering and (c) aspect ratio graph of two samples allocated by the randomized method.

Table 1. Correlation coefficients (R) of each pair-matched tooth using 3 different methods of sampling.

Matched pairs	Group 1 (randomized)	Group 2 (radiograph)	Group 3 (micro-CT)
Pair 1	0.537	0.258*	**0.928**
Pair 2	0.094*	0.370*	**0.942**
Pair 3	0.198*	0.484*	**0.935**
Pair 4	0.329*	0.633	**0.815**
Pair 5	0.534	0.554	**0.812**
Pair 6	**0.763**	**0.751**	**0.903**
Pair 7	0.491*	**0.823**	**0.899**
Pair 8	0.364*	0.416*	**0.936**
Pair 9	**0.972**	0.141*	**0.753**
Pair 10	**0.716**	0.138*	**0.911**

Very Strong (0.9 to 1.0) and Strong (0.7 to 0.9) correlations are depicted in bold letters, while asterisks show Weak (0.3 to 0.5) or Negligible (0.0 to 0.3) correlations.

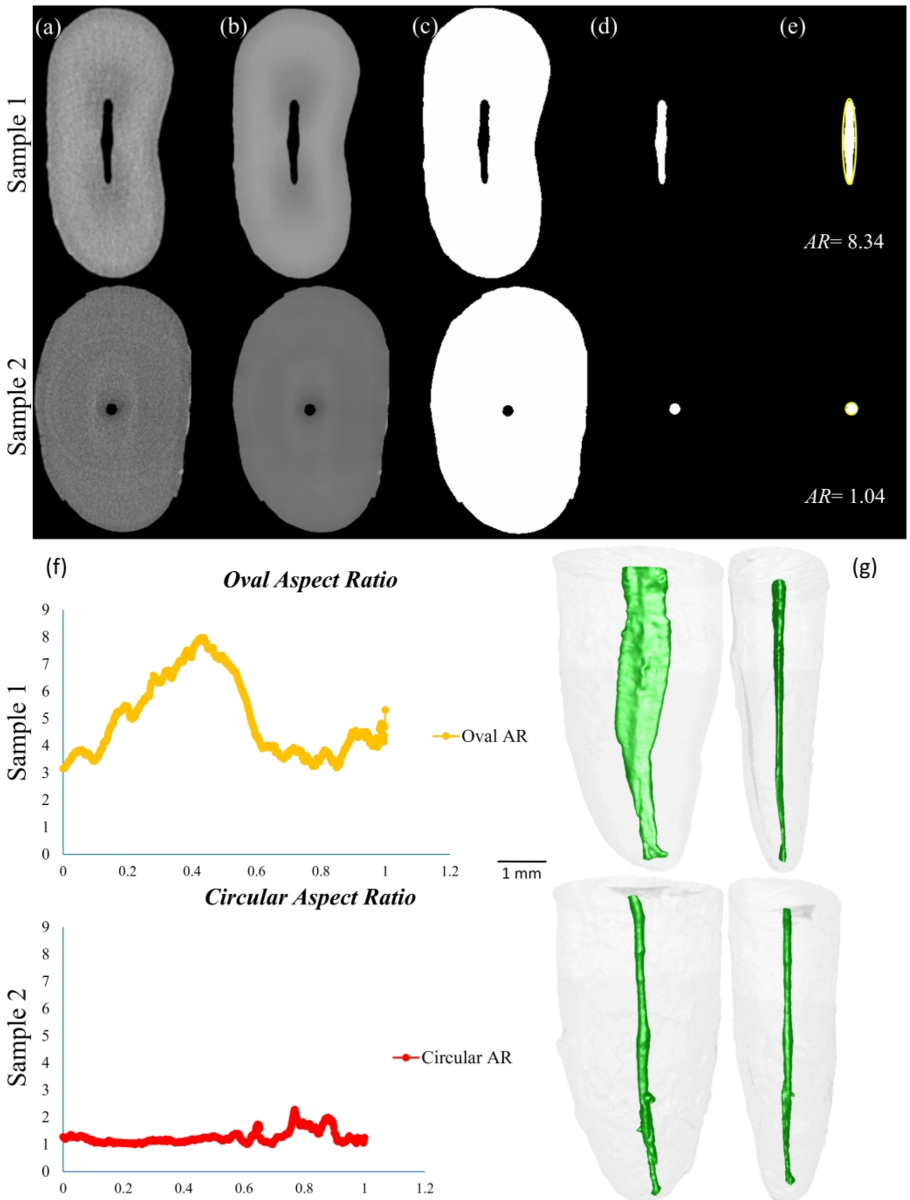

Figure 1. Micro-CT processing method used to select pair-matched mandibular incisors applied to representative samples having flattened (sample 1) and round-shaped (sample 2) root canals. (a) The cross-sectional images were submitted to (b) non-local means filter to reduce noise and (c) segmented. Then, (d) the canals were binarized and (e) the aspect ratio calculated and (f) plotted into graphs. (g) 3D models of the root and root canal of each specimen were also created and qualitatively evaluated in both buccal and proximal directions.

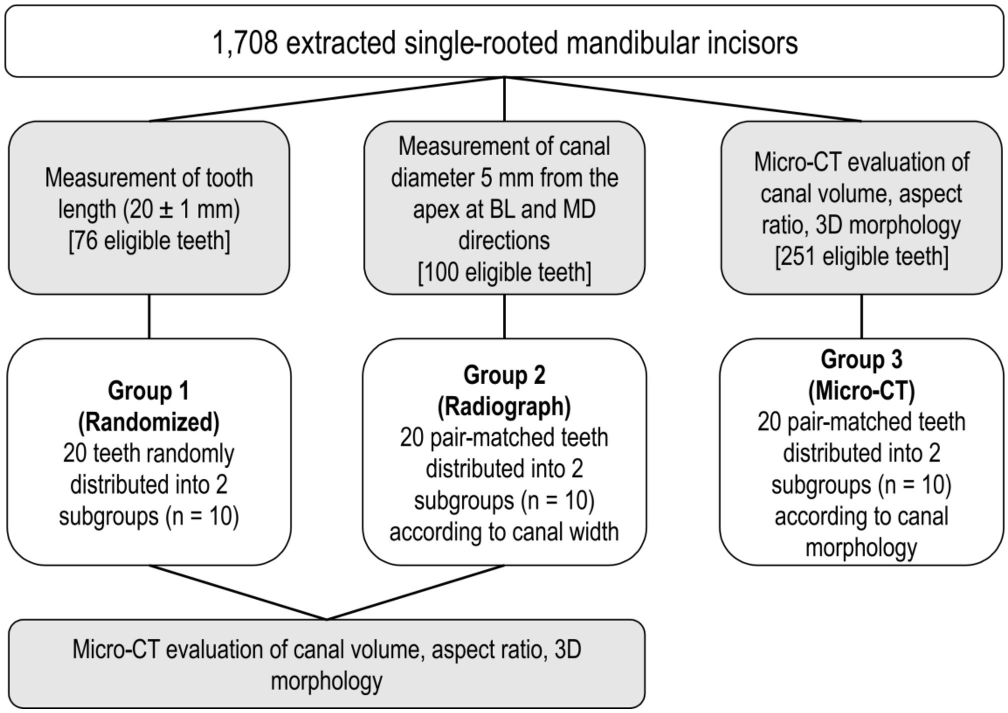

Figure 2. Flowchart of the methodology.

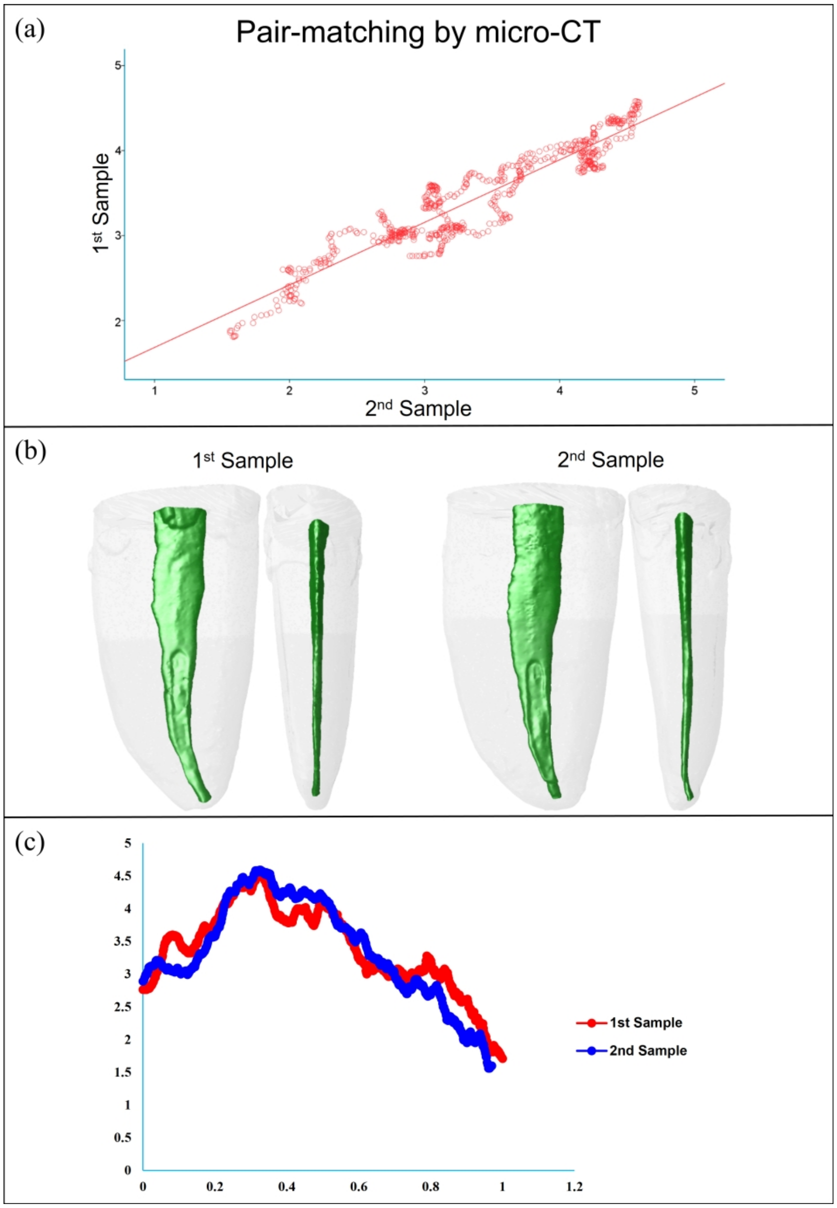

Figure 3. (a) Graph of correlation coefficients, (b) 3D rendering and (c) aspect ratio graph of two samples pair-matched based on micro-CT method.

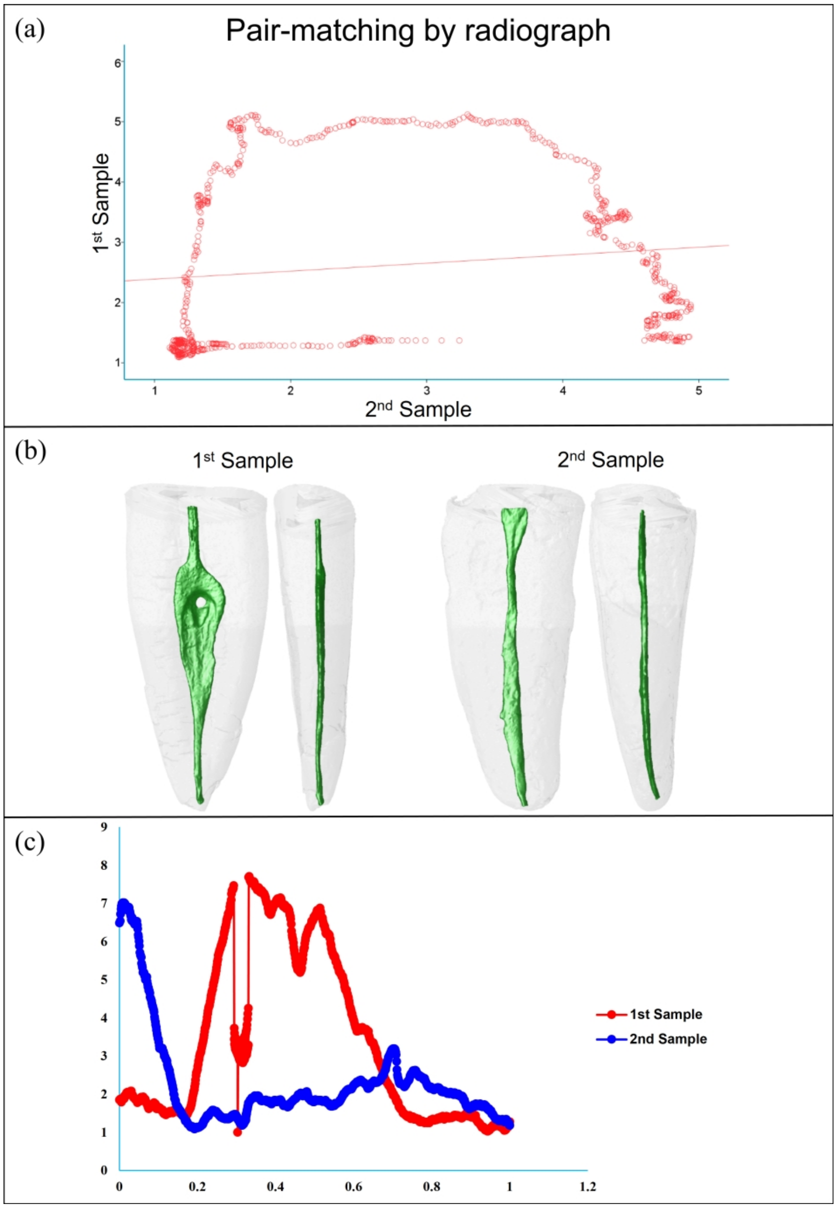

Figure 4. (a) Graph of correlation coefficients, (b) 3D rendering and (c) aspect ratio graph of two samples pair-matched by the radiographic method.

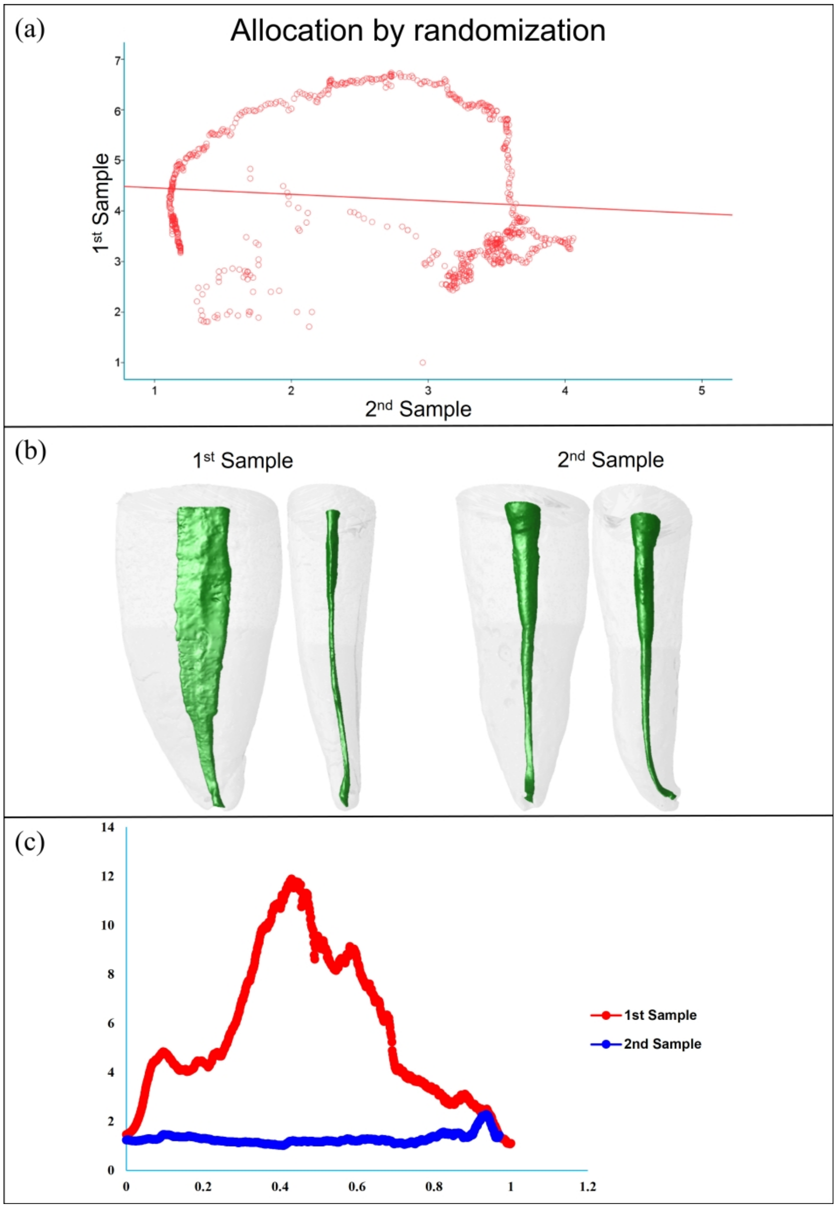

Figure 5. (a) Graph of correlation coefficients, (b) 3D rendering and (c) aspect ratio graph of two samples allocated by the randomized method.