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Removal of the apical one-third of the root improves the fixation process of the dental pulp in teeth

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The fixation process in histology is an important process that will eventually determine the quality of the histology slides. Fixation of the dental pulp involves the entry of the fixative agent into the dental pulp through the root canals that are found at the apical part of the root. The objective of this study was to evaluate the effectiveness of four methods of tooth preparation (teeth cut longitudinally or cervically, removal of apical third of root and whole uncut teeth) on the fixation process of the dental pulp for producing high quality histology slides. Among the four methods of tooth preparation, removal of the apical one-third of the root produced high quality histology slides as fixation of the dental pulp was improved and hence, preservation of the pulp’s architecture and contents were achieved. It is concluded that removal of the apical one-third of the root improves the fixation process of the dental pulp in teeth.

Keywords: Dental pulp, Fibroblasts, Fixation, Histology, Light microscopy, Odontoblasts, Subodontoblasts

Introduction
The tooth is the hardest tissue in the body and consists of both types of tissues; hard and soft. The hard tissues consist of enamel, dentine, and cementum, while the soft tissue is found in the inner part of tooth and is known as the dental pulp. Prior to routine processing of the tooth for producing histology slides, an extracted tooth is immersed in a fixative to prevent degradation of the dental pulp. However, the main challenge for fixation of the dental pulp is that the fixative can only get access to the dental pulp through the narrow opening of the root canal at the apical region of the root. It is crucial for histology slide production that the pulp is completely fixed, as this will ensure that the architecture and cellular details of the dental pulp is well preserved and will prevent degradation of the dental pulp. The dental pulp has been used for various types of research purposes such as immunological staining to identify specific proteins within the tissue or, in more recent times, associated or combined with molecular biology techniques.

In most cases, histology slides of the dental pulp have been obtained using whole uncut teeth. It has been noted that the histology slides obtained using whole uncut teeth may not have been fixed well and this could have affected the quality of the histology slides; among the factors affecting the dental pulp are distortion of pulpal architecture (e.g. pulp shrinkage) and loss of the morphological features of the pulpal cells. In addition, the time taken to demineralize a whole uncut tooth can take up to about 6 months or more; therefore, many studies have attempted to reduce the time taken for demineralization of the teeth by modifying the method and the solutions involved in the demineralization process.

The fixation and decalcification processes involving the use of various types of fixation/demineralizing solutions and the methods associated with the use of these solutions have been very well studied. However, to our knowledge, the effect of tooth preparations prior to the fixation and demineralization processes on routinely prepared hematoxylin and eosin (H & E)-stained histology slides has not been studied. Therefore, the aim of this study was to evaluate the effect of different types of tooth preparations (before placing the tooth in fixative and tooth
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decalcifying agents) on the quality of routinely prepared
histology slides.

Materials and Methods
The teeth included in this study consist of premolars and
third molars with complete root apaxes that were either
unaffected or only slightly affected by caries and those
requiring extraction for orthodontic purposes. All of the
patients involved in this study had complete dental records
and gave consent for their teeth to be extracted and used
for the study. The present study was approved by the
Ethical Committee of the Faculty of Dentistry, University
of Malaya. The extracted teeth were collected at the
Department of Oral and Maxillofacial Surgery, Faculty
of Dentistry, University of Malaya. Following extraction,
the extracted teeth were rinsed with 0.1M phosphate buff-
ered saline (PBS), stored in a separate bottle containing
PBS and immediately transferred to the laboratory. Forty
teeth were collected for this study and they were divided
into four groups consisting of ten teeth per group. The
methods for tooth preparation, as shown in Fig. 1, were as
follows: (i) tooth split longitudinally at the midline: a line
was drawn on the buccal surface of an intact tooth, and
sectioning was carried out slightly away from the drawn
line; (ii) removal of the apical third of the root: a horizontal
line was drawn at the apical third of the root before sec-
tioning; (iii) tooth cut along the cervical line: a horizontal
line was drawn on the cervical region of the interprox-
imal surface, and sectioning was carried out to remove the
upper portion of the crown; and (iv) whole uncut tooth.
Teeth that were collected for the whole uncut teeth method
were immediately placed in 10% neutral buffered formalin
for fixation (24 h) while teeth collected for the other three
methods were cut accordingly before being placed in 10%
neutral buffered formalin for 24 h. The teeth were cut
using an annular diamond blade of 300 μm thickness and
17.5 cm diameter. The diamond blade was attached to the
microslicer cutting machine (Malven, England) and the
teeth were cut with a continuous flow of PBS to prevent
overheating of the hard and soft tissues of the teeth.

Following fixation of the teeth in 10% neutral buff-
ered formalin for 24 h, all teeth were immersed in formic
acid (5%), pH 2.3 to decalcify the teeth. The formic acid
solution was changed every 3 days and the decalcification
process was continued until radiographic evidence showed
complete decalcification. In general, the time taken for
complete decalcification of the whole uncut teeth was
slightly longer when compared to 2 weeks required for
the teeth that were cut either longitudinally in the mid-
line, across at the cervical line or at the apical one-third
of the root. Following this step, the teeth were subjected
to routine processing for histological examination before
being embedded in paraffin wax (Appendix 1). Slices were
cut 5 μm thick and stained with hematoxylin and eosin
(Appendix 2). The sections were then checked under a
light microscope (Olympus CHK-B145) which was con-
ected to a computer at 4 to 100× magnifications.

Five best sections from each group were chosen for
further analysis. A quantitative scoring method was used to
obtain scores based on the following four factors: (a) Pulp
shrinkage, (b) Clarity of cell morphology, (c) Clarity of
cell staining, and (d) Organization of pulp zones. A score
of 1–10 was given for each of the factors. The average
score from the five sections for each factor was taken for
statistical analysis. At first, the sections were scored by one
examiner. Following this, three sections from each group
were re-examined by the same examiner and a different
examiner. Intra-examiner and inter-examiner reliability
were tested and found to be very good (0.89 and 0.81;
intra-examiner and inter-examiner reliability coefficient,
respectively).

A semi-quantitative analysis was also conducted to give
an overall score for the five sections examined from each

Figure 1 Four methods of tooth preparations used for
preparing histology slides. (a) tooth cut longitudinally at the
midline, (b) removal of the apical third of the root, (c) tooth
cut at the cervical line and (d) whole uncut tooth.
group, and the following scores were used for each of the factors: + below average, ++ average, +++ good, +++ excel-
lent. The mean score from five sections for each group
were compared by one-way ANOVA on ranks followed
by Tukey’s test. A \( p \) value of <0.05 was considered statis-
tically significant.

Results
Examination of the histology slides from whole uncut
teeth revealed that the pulp tissue seemed to be intact
with minimum shrinkage but, in some slides, the cellular
characteristics of the pulp cells were affected as the cel-
lar details were not well preserved (Fig. 2 (a1 and a2)).
Histology slides obtained from the teeth cut longitudi-
nally or at the cervical line often showed torn pulp tissue
which could reflect the cutting effect. The pulp underwent
shrinkage and pulp zones were not well demarcated. (Fig.
2 (b1 and b2) and 2(c1 and c2)). The cellular details of the
pulpal cells were also affected as the cells were not clearly
defined, well stained, or prominent. The histology slides
obtained by these two methods received the lowest score
(Fig. 4, Table 1). The method that involved the removal
of the apical one-third of the root region seemed to be
the most suitable method, as almost no pulp shrinkage
was observed. In addition, the cellular details of the den-
tal pulp seemed to have preserved satisfactorily (Fig. 3).
At a higher magnification, the different zones in the pulp
(consisting of odontoblasts, cell-free zone and the cell-
rich zone) were clearly demarcated as shown in Fig. 3.
The histology slides obtained from this method received
the highest score (Fig. 4, Table 1). The scores received for
this method were significantly higher (\( p < 0.05 \)) than the
methods for uncut teeth or for teeth that were cut longitudi-
nally or cervically (Fig. 4).

The quantitative assessment of histology slides pre-
pared by the four methods, as shown in Fig. 4, revealed
that the scores for the quality of the histology slides were
the highest when the apical one-third of the root was
removed, followed by the whole uncut teeth method. The
score obtained for the whole uncut teeth preparation was

![Figure 2 Photomicrograph of hematoxylin and eosin-stained sections obtained from three methods of tooth preparation; whole
uncut tooth (a1 (4x magnification) and a2 (10x magnification)), tooth cut longitudinally in the midline (b1 (4x magnification) and
b2 (10x magnification)), and across at the cervical line (c1 (4x magnification) and c2 (10x magnification)).](image-url)
Table 1 Light microscope assessment (semi-quantitative) of histological sections prepared from four methods of tooth preparation (n=10 for each group)

<table>
<thead>
<tr>
<th>Method of tooth preparation</th>
<th>Pulp shrinkage</th>
<th>Clarity of cell morphology</th>
<th>Clarity of staining properties</th>
<th>Organization of pulp cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncut tooth</td>
<td>+++ (6.9/10)</td>
<td>+++ (6.9/10)</td>
<td>+++ (6.8/10)</td>
<td>+++ (7/10)</td>
</tr>
<tr>
<td>Tooth cut at cervical</td>
<td>++ (5/10)</td>
<td>+++ (6/10)</td>
<td>+++ (5.9/10)</td>
<td>++ (5/10)</td>
</tr>
<tr>
<td>Tooth cut longitudinally</td>
<td>++ (5/10)</td>
<td>+++ (6/10)</td>
<td>+++ (5.9/10)</td>
<td>++ (5/10)</td>
</tr>
<tr>
<td>Removal of apical one-third</td>
<td>++++ (8/10)</td>
<td>++++ (6.1/10)</td>
<td>++++ (7.9/10)</td>
<td>++++ (8.2/10)</td>
</tr>
</tbody>
</table>

Assessment was based on a semi-quantitative scoring method; + below average, ++ average, +++ good, ++++ excellent. Average score from the quantitative method is shown parenthetically.

Discussion

One of the crucial steps in producing histology slides involves the fixation process, and it is therefore important to ensure that the fixative agents are able to thoroughly penetrate the dental pulp. The pulp chamber is enclosed by hard tissue in the crown and in the root, with an opening at the apical end of the root. The narrow opening at the apical end of the root may make it difficult for the fixative...
agents to completely penetrate and fix the dental pulp. This point was evident in this study, where pulp shrinkage was observed in some histology slides prepared using whole uncut teeth (Fig. 2 (a1 and a2)). Although the standard time used for routine fixation of soft tissues (24 h) was applied in this study, it could be that extending the fixation time may have given more time for the fixative to penetrate the dental pulp due to the narrow opening at the apical end of the root.

In order to improve the fixation process, there have been attempts to remove the hard tissue overlying the pulp chamber. The method was applied by Vogsayan and coworkers on animal and human teeth, and it was reported that the likelihood of the pulp tissue being damaged was quite high. This method was also employed by Keklikoglu and Akinci, who compared three different types of embedding materials for routine staining with hematoxylin and eosin. It was reported that, although removal of the coronal hard tissue was conducted very carefully, it was still very difficult to avoid pulp exposure and this sometimes resulted in the pulp tissue being torn.

The method of cutting the teeth longitudinally is commonly carried out for investigating the morphology of root canals and also in studies involving translucent dentine. It was reported that cutting teeth longitudinally quite often results in some form of damage to the pulp tissue. Although this method may be used for studying hard tissues of the teeth or the morphological pattern of the root canals, it might not be suitable for studies involving the dental pulp. This was evident in our findings, where histology slides obtained from teeth cut longitudinally or cervically often resulted in poor quality histology slides due to the damage of the pulp tissue.

The results obtained in this study revealed that fixation of the uncut teeth fared better than teeth that were cut longitudinally or along the cervical line. This could be due to the process of cutting the teeth, which often resulted in some damage to the pulp tissue, although great care was taken as not to expose the dental pulp. In addition, for teeth that were cut longitudinally or cervically, there was more contact between the decalcifying agent and the dental pulp tissue, and this could have caused further damage to the dental pulp, contributing to poor staining of the pulp tissue. To minimize the damaging effects of the decalcifying agent to the soft tissue of the dental pulp, 5% formic acid was used in this study, although percentages of up to 25% have been shown to have shortened the decalcification time with no deleterious effects on the pulp tissue.

Of the four methods, cutting the teeth at the apical third region of the root proved to be the best method, as evidenced in both the qualitative and semi-qualitative assessments of the histology slides. The histology slides produced with this method showed that the cells were well stained, prominent, and the natural architecture of the pulp was well preserved. This technique had also been used by Murray and coworkers, who studied age-related changes of the dental pulp cells and dentine thickness. The histology slides were used for cell counting of the odontoblasts, subodontoblasts, and fibroblasts of the dental pulp at the crown and root region. This method was also used recently by Sulinda and coworkers to study the morphological changes of age-related dental pulp cells. Removal of the apical one-third of the root region, where the pulp canal is the narrowest, would allow better penetration of the fixative agents into the dental pulp, thereby permitting complete fixation of the pulp tissue.

It is important in studies involving cellular morphology that the tissue involved undergoes complete fixation so that the cellular clarity is maintained closest to that of the living state. Fixation of the dental pulp can be challenging as the pulp is enclosed by hard tissue and the chemical substance used for fixation of the dental pulp can only enter the pulp through the narrow opening at the apical foramen of the root. When the apical one-third of the root region is removed, more of the chemical substance is allowed to enter and penetrate into the pulp cavity, as the diameter of the root canal is wider which will greatly enhance the chances of the dental pulp being completely fixed without damaging the architecture and contents of the pulp tissue.

Funding
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### Appendix 1 Tissue processing schedule

<table>
<thead>
<tr>
<th>Station</th>
<th>Reagents</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70% Alcohol</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95% Alcohol</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>95% Alcohol</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>100% Alcohol</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>100% Alcohol</td>
<td>1½</td>
</tr>
<tr>
<td>6</td>
<td>Xylene</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>Xylene</td>
<td>1½</td>
</tr>
<tr>
<td>8</td>
<td>Xylene</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>Wax (46–48 °C)</td>
<td>1½</td>
</tr>
<tr>
<td>10</td>
<td>Wax (56–58 °C)</td>
<td>3</td>
</tr>
</tbody>
</table>

### Appendix 2 Hematoxylin and eosin staining schedule

<table>
<thead>
<tr>
<th>Station</th>
<th>Reagents</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Xylene I</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Xylene II</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>100% Ethanol</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>95% Ethanol</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>70% Ethanol</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Running water</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Harris hematoxylin solution</td>
<td>15–20</td>
</tr>
<tr>
<td>8</td>
<td>Running water</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>Acid alcohol</td>
<td>2 dips</td>
</tr>
<tr>
<td>10</td>
<td>Running water</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>Potassium acetate</td>
<td>6 dips</td>
</tr>
<tr>
<td>12</td>
<td>Running water</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>80% Ethanol</td>
<td>3 dips</td>
</tr>
<tr>
<td>14</td>
<td>Eosin solution</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>95% Ethanol</td>
<td>4 dips</td>
</tr>
<tr>
<td>16</td>
<td>95% Ethanol</td>
<td>4 dips</td>
</tr>
<tr>
<td>17</td>
<td>100% Ethanol</td>
<td>4 dips</td>
</tr>
<tr>
<td>18</td>
<td>100% Ethanol</td>
<td>4 dips</td>
</tr>
<tr>
<td>19</td>
<td>Xylene I</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>Xylene II</td>
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</tr>
<tr>
<td>21</td>
<td>Xylene III</td>
<td>3</td>
</tr>
<tr>
<td>22</td>
<td>Mount in DPX</td>
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</table>