UNIVERSITI SAINS MALAYSIA

USM Short term grant final / completion report
304/PPSK/6131185

Human molar dentinal tubules and its odontoblast process. A combined investigation using variable pressure scanning electron microscope and image analysis.

By

Dr Farid Bin Che Ghazali
Principle researcher

Prof. Madya Dr Syed Hatim Noor @ Nyi Nyi Naing
Co researcher

En Jamaruddin Mat Asan
Technologist

School of Health Sciences (PPSK),
Health Campus, 16150. Universiti Sains Malaysia,
Kubang Kerian. Kelantan Darul Naim.

December 2004

Email: farid@kb.usm.my

Author Keywords and Abbreviations: Dentine, Dentinal tubules, Odontoblast processes and Variable pressure scanning electron microscopy (VPSEM).

Abstract
Electron microscopy studies of the dentinal tubules are mainly investigated using the high vacuum conventional scanning electron microscope (SEM). We aimed to investigate its morphology using the variable pressure SEM Three almost caries free intact adult human molar tooth were prepared as unembedded demineralised whole dentine preparations that were initially chemically fixed with 10% formalin for 24 hours. They were demineralised in 10% EDTA solution for 4 weeks following, which they were than section into two equal halves. The uncoated partially demineralised dentine half tissue was inspected systematically under a LEO VPSEM using the peltier cooling sub stage at 15 Pascal pressure with an accelerating voltage of 15 kV. An occlusal and longitudinal observation was made from its occlusal surface to the pulpal border. Both low and high magnifications images were prepared from each specimen observed.

Introduction

Dentine is a biological mineralised tooth structure that is densely perforated with dentinal tubules that extend from the pulp chamber to the enamel-dentine junction. The presence of dentinal tubules is an attributing factor to dentine permeability (Thomas, 1985). There are numerous literatures descriptions of odontoblast processes in dentine tubules (Ten Cate, 1967; Brannstrom and Garberoglio, 1972; Garberoglio and Brannstrom, 1976). These earlier studies measured the diameter and number of the dentinal tubules in animal and human dentition using both the light and the conventional scanning electron microscope but still the extent of the process and the volume of the dentinal tubule it occupies especially in human tooth model are some features that still remain until now controversial. At present the odontoblast layer is being suggested as a barrier. Little knowledge is known now to suggest the mechanism orchestrated by this layer. To understand the barrier there is a need to acknowledge the functional odontoblast cellular kinetics that maintains its intactness as a layer and its ultrastructural significant. This will further understanding of the role of the intercellular junctional complexes of the odontoblast layer towards exogenous permeation of fluid and substances.

In mineralised dentine there are thousands of microscopic tubules that extend from the pulp-dentine border to the enamel-dentine junction (EDJ) or cement-dentine junction (CDJ). A morphometric assessment of the cross sectional area of coronal dentine occupied by dentinal tubules in human third molars gave an incidence of 22,000 dentinal tubules per square millimetre near the enamel-dentine junction while midway between the pulp chamber and the enamel-dentine junction the number of tubules was 37,000 mm^{-2}. Closer to the pulp the number of dentinal tubules was 48,000 mm^{-2} (Dourda, Moule and Young, 1994). Hence the numbers of tubules present per square millimetre increased from the

EDJ to the pulp (Mjor & Nordahl, 1996; Dourda, Moule & Young, 1994; Berkovitz, Holland & Moxham, 1992;).

Variable pressure scanning electron microscope is new introduction to high-resolution microscopy. The microscopical investigation using the variable pressure scanning electron microscope (VPSEM) is based on the inquisitive recognition of the tissue morphological characteristics in their natural hydrated state. No prior knowledge in staining regents or coating is needed. In conventional high vacuum scanning electron microscopy, specimens need to be dehydrated in critical drying and then coated with a layer with conductive material like gold to dissipate charge build up. Thus VPSEM allows image to be studied uncoated and fully hydrated and reduced sample preparation requirements, in such a situation its application in particular to forensic science applications have been significantly noted.

Hals1983, and Dyngeland *et al.,* 1983, described the presence of wide diameter 'giant tubules' that was observed in human coronal dentine. The presence of these giant tubules was supported by a series of paper that was presence in animal and human tooth dentin model but a review of the literatures showed that these studies were largely investigated in unerupted anterior tooth. In a pilot study Farid et al, (2001) studied on five caries free adult human molar with special emphasis to its dentinal tubules diameter and numbers located at the middle part of the coronal end of the pulp chamber. The statistical summary showed that the mean and standard deviation were: area 4.88 ± 2.43, length 3.22 ± 0.94, breadth 2.08 ± 0.58, perimeter 9.93 ± 3.22, and equivalent diameter 2.41 ± 0.62 which indicates normally distributed data.

Objective

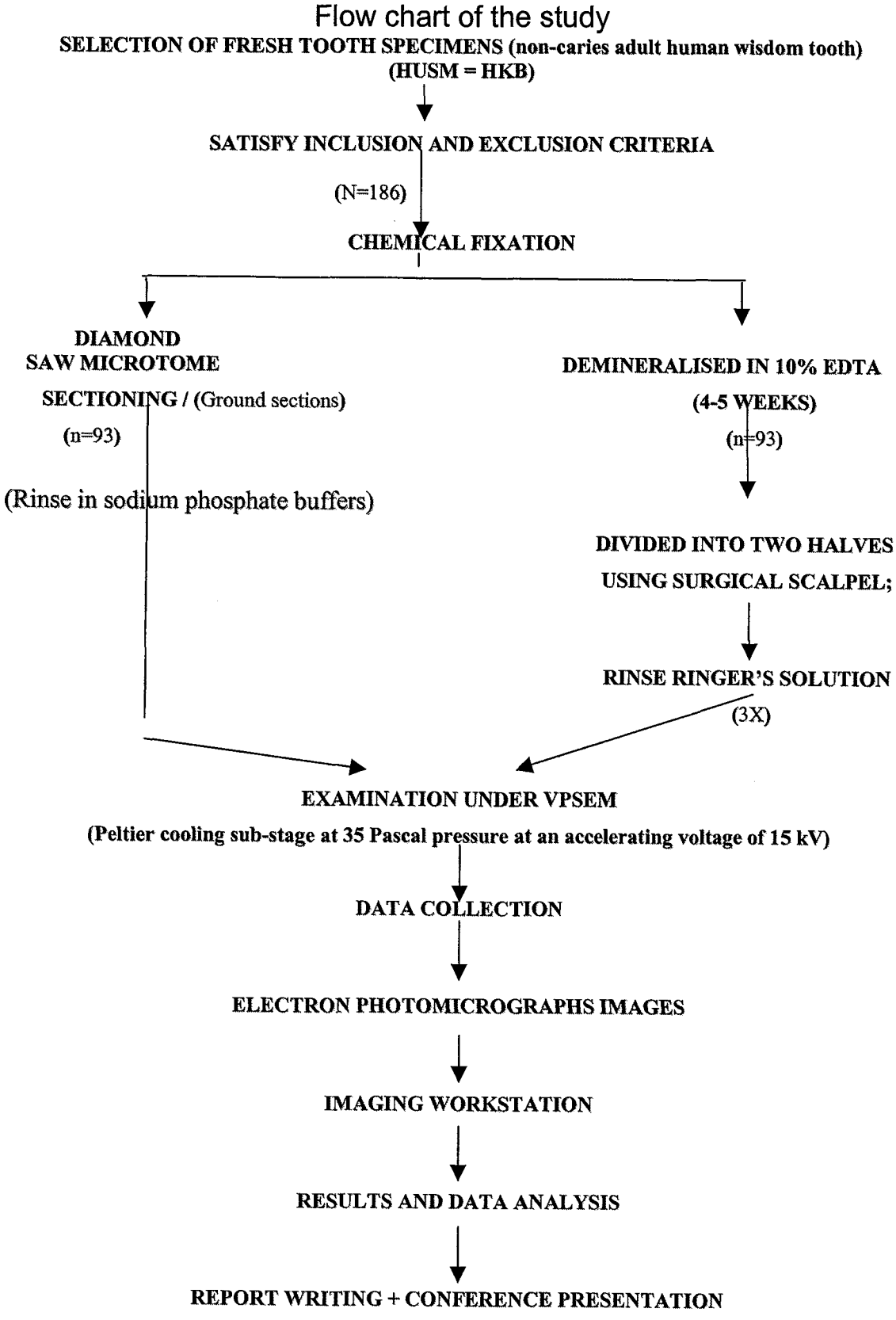
1. To gain insights of use to oral biology especially to the understanding of dentine sensitivity.
2. To observe human tooth dentinal tubule density and diameter as measured at various distances from the enamel dentine junction to the predentine-pulp interface.
3. To observe the dentinal tubule density and numbers observed under the VPSEM in almost its hydrated natural condition.
4. To observe extension of the odontoblast process in its almost hydrated natural condition in unerupted mandibular tooth /wisdom tooth'.
5. To observe the different levels of mineralisation of dentine using backscattered electron-imaging (BSE) technique of the VPSEM.
6. To compare ground and demineralised dentinal tubule's preparations measurements.

Methodology

This study was focused to recognize the natural hydrated anatomical overview of the dentine morphology in non-caries molar human tooth preparations with special emphasis to its dentinal tubules diameter, incidence and numbers located over the whole length of the dentine proper. The dentinal tubules spatial arrangement and diameter will be examined using a variable pressure scanning electron microscope LEO 1455VP and the dentine tooth preparation will be prepared without the tedious conventional scanning electron microscope tissue preparation protocols. The criteria for sample inclusion are normal healthy non-painful fresh tooth wherelse exclusion of sample are caries, painful, old extracted tooth. A minimal number of 30 non-caries adult human mandibular third molar teeth will be selected. The tooth will be collected fresh from HUSM operating theatre or HKB dental surgeries. The teeth will be selected from those indicated for wisdom tooth removals especially those that is indicated for orthodontics requisite. The investigator will not make the decision for extraction. The teeth will be chemically fixed in full strength Karnovsky's immediately after surgical removal for 24 hours, than either sectioned with a diamond rotary saw microtome as ground sections or demineralised in 10% EDTA at pH 7.3 for 4 weeks, with regular changes of the demineralisation solution. Based from previous works by the 4th week enamel will be macroscopically absent leaving occlusal dentine exposed. The demineralised dentine will be divided into two equal halves using a surgical scalpel; each halves will then be rinsed with repeated changes of Ringer's solution. Each demineralised halves and ground sections will be examined under the VPSEM, using its peltier cooling sub-stage at 35 Pascal pressure at an accelerating voltage of 15 kV. They will be examined from their occlusal surface to the pulpal border. Photomicrographs images of the dentinal tubules will be transferred to Leica imaging workstation to enable accurate location of the study areas. Grey image processing was used to extract dentinal tubules. Grey level threshold will be set to detect only the tubules. Binary image processing will be used to remove little artefacts. Resultant image will be measured and colour-coded in accordance to histogram classes. Statistical results for area, length, breadth and diameter will be expected to be derived and tabulated.

Imaging Methodology:

Electron microscopy studies of the dentinal tubules are mainly investigated using the high vacuum conventional scanning electron microscope (SEM). We aimed to investigate its morphology using the variable pressure SEM The specimens will be examined using a LEO 1455VP Variable Pressure Scanning Electron Microscope (VP SEM). In variable pressure mode, neutralization of charge build up in insulating samples was achieved by adjusting chamber pressure (range from 1Pa to 400Pa). In general, greater pressure will be used for more insulating specimens. Without variable pressure mode, the specimens will be difficult to be examined using conventional high vacuum SEM. In conventional high vacuum, specimens need to be coated with a layer with conductive material like gold to dissipate charge build up.


Flow chart of the study
SELECTION OF FRESH TOOTH SPECIMENS (non-caries adult human wisdom tooth)
(HUSM = HKB)
SATISFY INCLUSION AND EXCLUSION CRITERIA
(N=186)
CHEMICAL FIXATION
DIAMOND
SAW MICROTOME
SECTIONING / (Ground sections)
(n=93)
(Rinse in sodium phosphate buffers)
DEMINERALISED IN 10% EDTA
(4-5 WEEKS)
(n=93)
DIVIDED INTO TWO HALVES
USING SURGICAL SCALPEL;
RINSE RINGER'S SOLUTION
(3X)
EXAMINATION UNDER VPSEM
(Peltier cooling sub-stage at 35 Pascal pressure at an accelerating voltage of 15 kV)
DATA COLLECTION
ELECTRON PHOTOMICROGRAPHS IMAGES
IMAGING WORKSTATION
RESULTS AND DATA ANALYSIS
REPORT WRITING + CONFERENCE PRESENTATION

Sample size calculation

$$N=\frac{2\sigma^2 (Z\alpha+ Z\beta)^2}{\Delta^2} \cong$$

$$N=\frac{2X (2.43)^2 (1.96+0.84)^2}{(1.0)^2} = 92.58$$

=93 per group

($Z\alpha$= 1.96, $Z\beta$ = 0.84, σ = 2.43, Δ = 1.0)

Reason for selecting just mandibular third molar tooth.

1. It's the last tooth to erupt into the oral cavity hence it is expected that the selected tooth specimen will be of minimal or caries free.
2. The tooth will be least exposed to mastication activities hence attrition and erosion that leads to crown fracture and present of micro leakage should be minimal or absent.
3. Medical and dental history of consenting patients will be bettered assessed on the day of surgery.
4. Finally the tooth specimen can be obtained fresh.

Outcome:

1. By measuring the diameter of the tubules better understanding of dentine permeability in human model preparation (ground and demineralised) will be derived. Thus providing further background data for further scientific researches in dentine permeability and endodontics.
2. It wills enhanced better overview of the morphological changes as observed in dentine tissue especially in their hydrated state.
3. Promote mineralised tissue research especially in tooth ultrastructural hence thus to encourage collaborative research with overseas centres.
4. Transfer expertise in mineralised tissue and high-end resolution microscopy to University Sciences of Malaysia.

Results and Discussions

Demineralised dentine undergoes morphological shrinkage during normal dehydration post-demineralisation and thus provides a false value of its tubule density and diameter (Garberoglio and Brannstrom 1976). In our partially demineralised dentine preparations the tubules appeared intact and were mostly empty. Occasionally a cylindrical whitish tread like substance was observed transversing along the major length of the dentinal tubules they were suggestive of putative odontoblast processes. The dentinal tubule diameter measured at random was approximately within 4 υm to 5 υm in diameter. Summary statistics showed that mean and standard deviation were: area 4.88 ± 2.43, length 3.22 ± 0.94, breadth 2.08 ± 0.58, perimeter 9.93 ± 3.22, and equivalent diameter 2.41 ± 0.62 which indicates normally distributed data. No intratubular dentine was

observed, both in the coronal and pulpal ends. Arends *et. al.,* (1995) [1]; measured the diameter of the dentinal tubules at a distance of about 1.5 mm from the pulp and found that the diameter was affected by the critical point drying techniques regularly used in conventional scanning electron microscope. In this study, the change in diameter observed in the preparations was quite minimal. In conclusion VPSEM offers a promising horizon and new challenges for further understanding especially in the extent of odontoblast process and lamina limitans morphology within dentine structure.

Conclusion

Variable pressure scanning microscopy when used to study mineralised tissue such as dentine or bone, in their hydrated natural state and with the present of other soft tissues offers a very promising potential and new challenges for further probing and understanding of various controversies still lingering especially in the extent of odontoblast process and lamina limitans morphology within dentine structure.

References

- Arends, J., Stokross, I., Jongebloed, W. G., Ruben, J. (1995). The diameter of the dentinal tubules in human coronal dentine after demineralisation and air-drying. A combined light and SEM study. *Caries Res.* 29. 118-121.
- Berkovitz, B. K. B., Holland, G. R., Moxham, B. J. (1992). Secondary dentine and post eruptive features. In: A colour atlas and textbook of oral anatomy, histology and embryology. 2nd Eds, Wolfe Publishing Ltd. 142-143.
- Brannstroms, M., Garberoglio, R. (1972). The dentinal tubules and the odontoblast process; A scanning electron microscopic study, *Acta Odontol Scand* 30: 291.
- Dourda, A. O., Moule, A. J. and Young, W. G. (1994). A morphometric analysis of the cross sectional area of dentine occupied by dentinal tubules in human third molar teeth. *Int. Endod.J.* Jul. 27 (24): 184-189.
- Dyngeland T, G. Fosse, N.P, B. Justesen (1983), Histochemical study of giant tubule content in dentine of unerupted cow incisors. *Scand J Dent Res* 92, 177-182.
- Farid Bin Che Ghazali, Nyi Nyi Naing, Ismail Abdul Manan (2001). The coronal pulp chamber dentinal tubules. A combined investigation-using variable pressure scanning electron microscopy and imaging analysis. Accepted abstract proceeding Asean Medical Conference.
- Garberoglio, R., Brannstrom, M. (1976). Scanning electron microscopic investigation of human dentinal tubules. *Archs oral Biol.* 21. 355-362.
- Hals. (1983), Observations on giant tubules in human coronal dentin by light and micro-radiography. *Scand J Dent Res* 91, 1-7.

- Mjor, I.A. Nordahl, I. (1996). The density and branching of dentinal tubules in human teeth. *Archs oral Biol.* 41, (5), 410-412.
- Ten Cate, A. R. (1967). A histochemical study of the human odontoblast. *Archs oral Biol.* 12. 963-969.
- Thomas, H.F. (1985). The dentine-predentine complex and its permeability; Anatomical overview. *J. Dent Res.* 64 (spec no): 607-612.

Acknowledgement.
The authors would like to acknowledge that the funding of this research project was supported by a USM short-term grant: 3045/PPSK/6131217. And thank Dr Endang Purwani for providing the fresh surgically removed tooth that was used in this experiment.

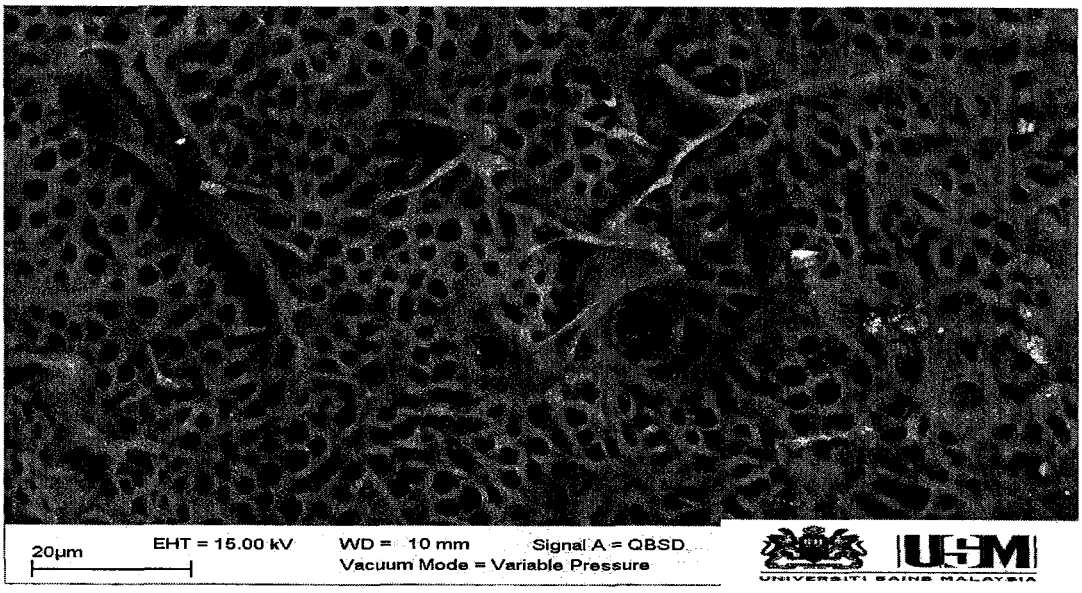


Figure I: Dentinal tubules orifices observed at the coronal pulpal end of the dentine preparation. No intratubular dentine was observed.

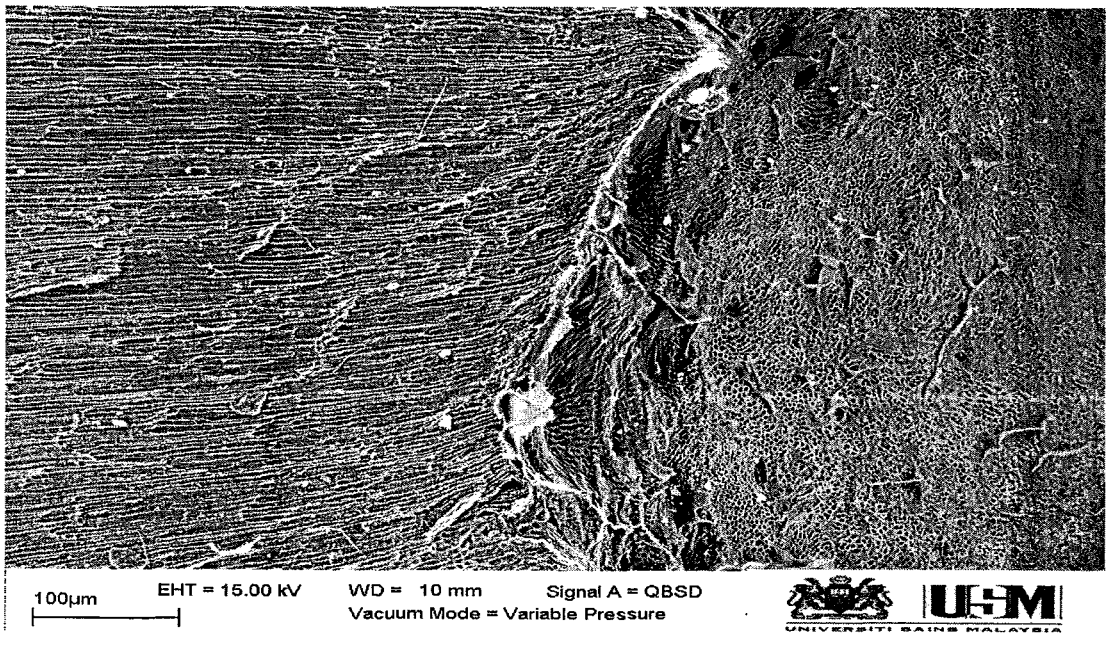


Figure II: Pulpal-predentine border showing the longitudinally running dentinal tubules and its orifice at the pulp end.

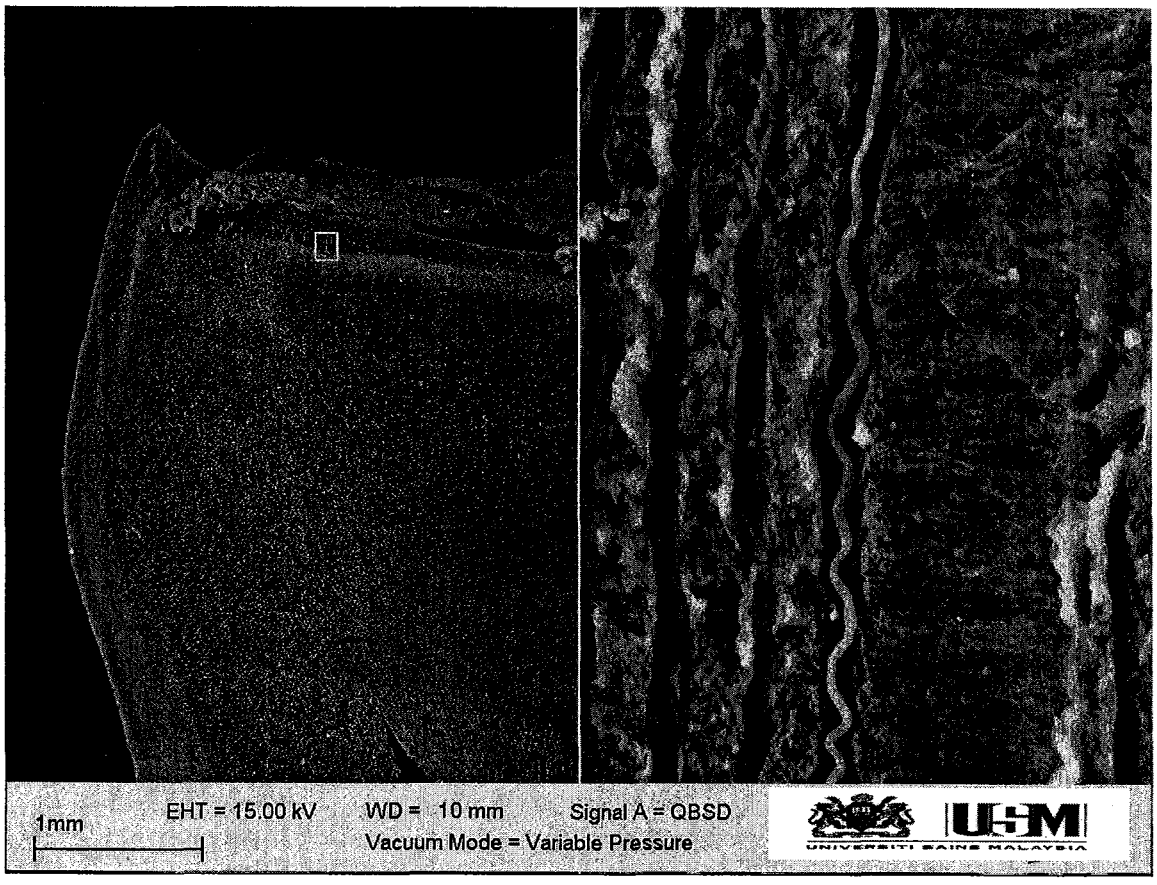
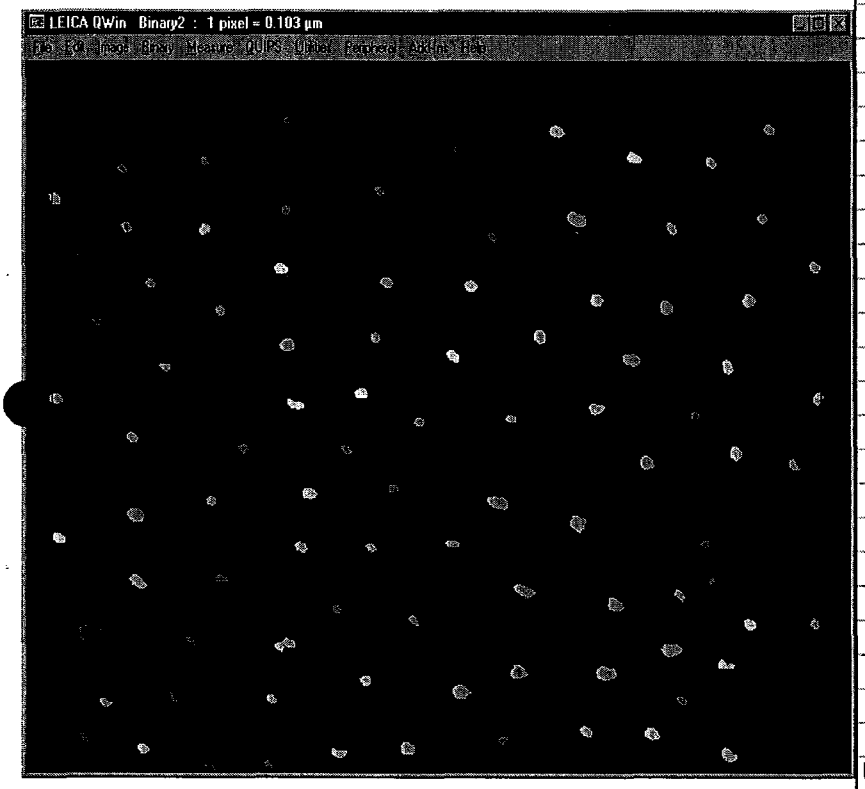
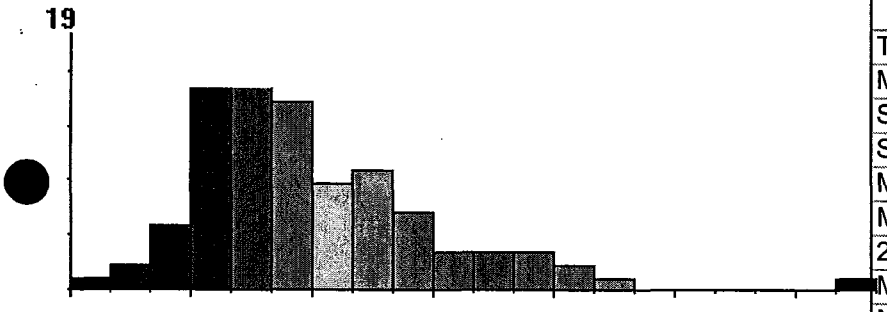


Figure III: Whitish tread like material suggestive of a putative odontoblast process observed in the coronal end of the dentine.

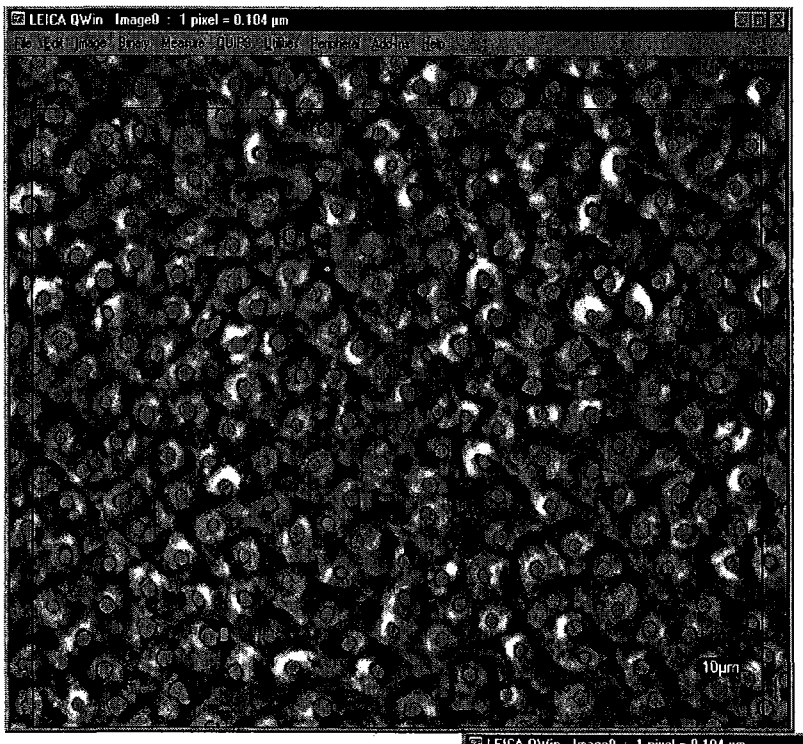
Feature Measurement



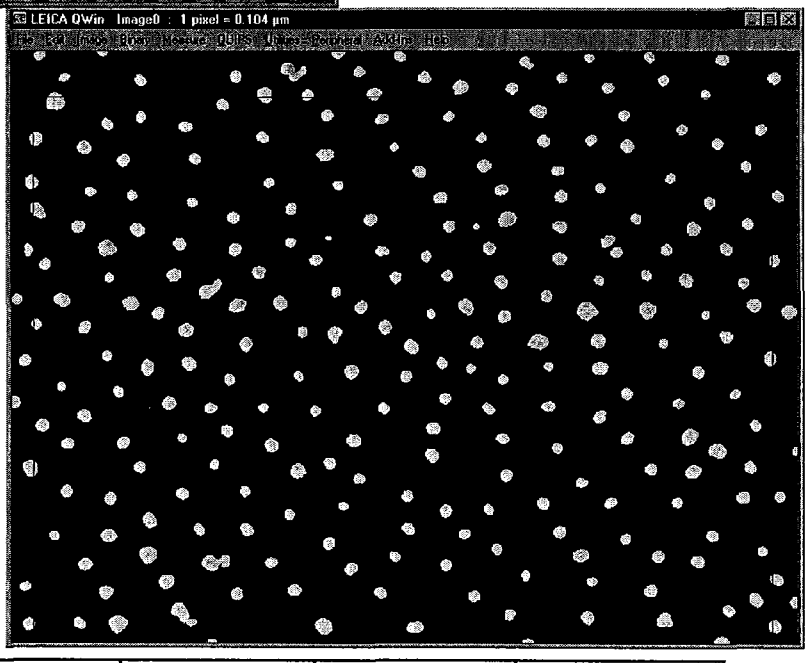


Bin	Area (µm²)	Count	Count (%)	
1	0	0.25	1	1.06
2	0.25	0.5	2	2.13
3	0.5	0.75	5	5.32
4	0.75	1	17	18.09
5	1	1.25	19	20.21
6	1.25	1.5	14	14.89
7	1.5	1.75	8	8.51
8	1.75	2	9	9.57
9	2	2.25	6	6.38
10	2.25	2.5	3	3.19
11	2.5	2.75	3	3.19
12	2.75	3	3	3.19
13	3	3.25	2	2.13
14	3.25	3.5	1	1.06
15	3.5	3.75	0	0
16	3.75	4	0	0
17	4	4.25	0	0
18	4.25	4.5	0	0
19	4.5	4.75	0	0
20	4.75	5	1	1.06
Undersize	0		Total Coun	94
Oversize C	0			
Total	140.54			
Mean	1.5			
Std Dev	0.75			
Std Error	0.08			
Max	4.93			
Min	0.12			
2-s Range	3.01			
Median	1.38			
Mode	1.13			
Skewness	1.45			
Kurtosis	3.44			
Features	94			
Spec. Area	7188.96			
Norm. Cou	13075.6			

Dentine-mid-lower-third



Area	377.722
Count	220
Area Fraction	0.053
Area Fill	0.055
Area Percent	5.254
Measure Frame	7188.965



Number	Area	Length	Breadth	Perimeter	Roundness
	(µm²)	(µm)	(µm)	(µm)	
Total	381.554	358.856	295.686	1105.991	248.25
Mean	1.734	1.631	1.344	5.027	1.128
Std Dev	0.674	0.366	0.251	1.022	0.091
Std Err	0.045	0.025	0.017	0.069	0.006
Max	4.446	3.807	2.263	11.523	2.234
Min	0.296	0.72	0.617	2.263	1.045
2-s Ran	2.697	1.465	1.004	4.088	0.365
Features	220				