Dental Materials Journal 26(3): 414−421, 2007

Development of New Software as a Convenient Analysis Method for Dental Microradiography

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Received April 12, 2006 /Accepted January 29, 2007

To the end of developing a convenient research tool to calculate the mineralization status of teeth in detail, a new program was developed using Visual Basic for Applications combined with Microsoft Excel 2004. To demonstrate the usefulness of this program, it was used to analyze tooth enamel mineralization after acid exposure. Transverse microradiography images (TMR) of specimens were digitalized with a charge-coupled device camera with a microscope (CCD camera) and a digital film scanner (FS). Subsequently, the mineral content profile of each specimen after de- and remineralization studies were calculated using the Angmar's formula. The newly developed program was applied to calculating the mineral loss (ΔZ), lesion depth (Ld), surface zone depth (SZd), and lesion body depth (LBd) of tooth specimens. In addition, the outer surface zone (OSZ), inner lesion body (ILB), and sandwich area (SA) between OSZ and ILB — which together constituted ΔZ — were calculated by the newly developed program. Data obtained with the newly developed program were in good agreement for both CCD camera and FS, indicating that the program was reliable for tooth enamel mineralization research studies.

Keywords: Transverse microradiography, Digital image analysis, Measurement of tooth enamel mineralization

INTRODUCTION

Presently, in caries research, an array of analytical techniques are available to determine the mineral content profile of dental hard tissue[1-3]. Amongst which, transverse microradiography (TMR) is used most widely[4]. In particular, quantitative contact microradiography is TMR whereby tooth slices are cut transversely to the labial surface of the tooth[5]. With this method, images of a tooth slice and an aluminum step wedge are made on film. Following which, the densitometry data obtained can be used to calculate the mineral content profile, expressed in Vol% or kg/m^3 using Angmar's formula[6].

The conventional method to measure the optical density of TMR images is line scanning with a microdensitometer with a slit of 20−30 μm and a width of 1−2 μm. Recently, more powerful scanning devices for TMR films and accompanying software for quantitative calculations have been introduced[5,7-10]. One such scanning device is the CCD camera which allows for two-dimensional scanning of TMR films of carious lesions[10]. Alternatively, a digital film scanner (FS) has a wider scanning area, and its accuracy of 4.7 μm is quite similar to that of a CCD camera at 5 μm, as reported by Arends and Bosch[4].

In previous studies[11,12], a commercially available software was used to calculate the mineral loss (ΔZ) and lesion depth (Ld) of tooth enamel. However, such commercial software is available only at a prohibitive price. Therefore, the first aim of the present study was to develop a new program to calculate these parameters using Visual Basic for Applications combined with Microsoft Excel 2004 — so that the new program can be used freely and widely by other researchers. In addition, this program was capable of calculating the surface zone depth (SZd), lesion body depth (LBd)[13], and three other parameters such as outer surface zone (OSZ), inner lesion body (ILB), and the sandwiched area (SA) between OSZ and ILB. The latter three parameters are not included in the commercial program. Second, to the end of verifying and validating the newly developed program, comparisons were made with a charge-coupled device camera with a microscope (CCD camera) and a digital film scanner (FS) using the same samples employed for calculation of tooth enamel mineralization.

MATERIALS AND METHODS

Figure 1 shows schematically the analysis procedure in this study.

Specimen preparation

Eight extracted, non-carious, human molars from different patients were used for the present study. The present study was approved by the Ethical Committee of the Hokkaido University Graduate School of Dental Medicine.

De- and remineralization specimens were made according to the procedures described by Saeki *et al.*[14]. Enamel surface of the crown segment was coated with Sticky Wax (Kerr, USA), with a 3 × 4 mm^2 window. Specimens were firstly demineralized in 0.01 M sodium acetate buffer (pH 4.0) at 50℃ for two days[14]. After demineralization,

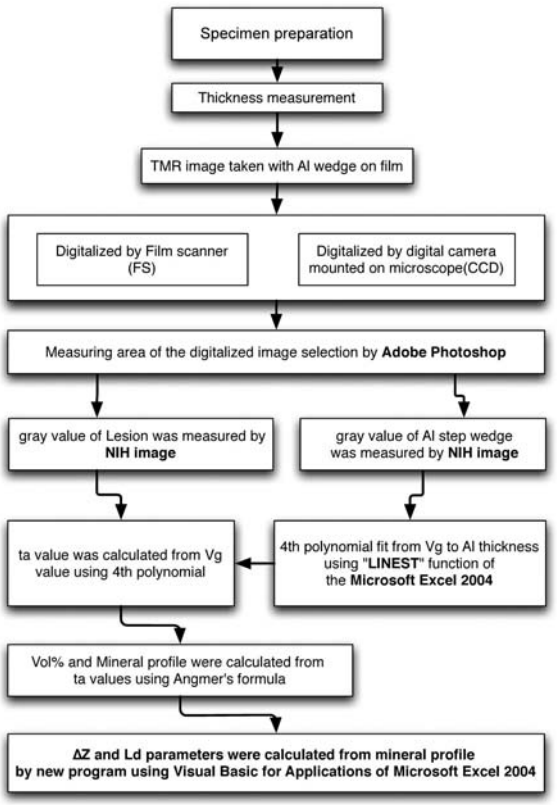


Fig. 1 Flow diagram of transverse microradiography analysis.

half of the window was coated with Sticky Wax. The other half of the window was remineralized by immersing in a remineralizing solution of 1.0 mM $CaCl_2$, 0.6 mM KH_2PO_4, and 0.1 M NaCl (pH 7.3) at 37℃ for two weeks with agitation by an agitator[14]. After demineralization and remineralization, Sticky Wax was removed with xylene and the specimen embedded in Rigolac (Nissin EM, Tokyo, Japan). In this manner, both the de- and remineralized areas were included in the same specimen from each tooth.

Longitudinal sections of 500-μm thickness were generated from each of the eight teeth using a low-speed saw (Isomet, Buehler, USA) under water coolant. All sections were reduced to a thickness of approximately 100 μm using #1000 and #2000 whetstones. Thickness of the specimens after polishing was measured by a vernier micrometer (CD-15CX, Mitutoyo, Japan).

Transverse microradiography (TMR) procedure

An aluminum step wedge (24 μm × 11 steps) and the specimen to be studied were fitted together side by side on a high-resolution X-ray film (Type 1A, Kodak, Rochester, USA). An X-ray machine using Ni-filtered CuKα radiation (Softex-CSM-2, Softex, Japan) was operated at 10 kV and 3 mA, as was so performed in a previous study[15]. Exposure time was 30 minutes, and the focus-specimen distance was 44 mm[14]. Films were then developed using a Kodak D19 developer (Kodak, Rochester, USA) under standard conditions.

Digitalizing transverse microradiography (TMR) images

1. Charge-coupled device camera with a microscope (CCD camera)

The film was mounted on a light microscope (BX50, Olympus, Tokyo, Japan) equipped with a CCD camera (DP12, Olympus, Tokyo, Japan). The microscope was set at ×10 objective magnification, and the illumination of the microscope was filtered by an LBD filter to correct the color temperature. Settings of the CCD camera were ISO 50 and 6500K white balance. Video signal generated by interlace scan CCD (DP12, 2048 × 1536 pixels) was digitalized with an 8-bit resolution (256 gray levels). Images were stored in TIFF format in dynamic memory. Subject area with the microscope was 3×10^6 pixels and 4×10^6 μm^2. Since the scanning area was limited by the CCD camera, digitalization of the specimens and the aluminum step wedge were performed separately.

2. Digital Film Scanner (FS)

Microdensitometric image analysis of developed X-ray films was performed with a system consisting of a digital film scanner (DIMAGE Scan Elite 5400, Minolta, Japan) and a personal computer (Power Macintosh G5, Apple, USA). Video signal generated by the image sensor — RGB 3-LINE CCD (5300 pixels/line) of DIMAGE — was digitalized with an 8-bit resolution (256 gray levels) using cold cathode fluorescent tube illumination. Digital video signal data thus obtained was temporarily stored in the dynamic memory (10M bytes) of the personal computer, and scanned images of 5232×7800 pixels were written into disk files in TIFF format for permanent storage. Area including the specimen and aluminum step wedge was 4×10^7 pixels and 9×10^8 μm^2.

Analysis of digital images

1. Curve-fitting procedure of gray values *versus* aluminum step wedge

Mean gray values of each aluminum step wedge were measured by NIH Image Ver 1.63 for Macintosh (developed at the US National Institutes of Health). Subsequently, the obtained values were plotted against the thickness of the aluminum wedge (Fig. 2). From the obtained data, a 4th degree polynomial formula curve[5,10] was derived by a least square method using the LINEST function of Microsoft Excel 2004 for Macintosh (Microsoft Incorporated, USA).

2. Measuring gray values at lesion sites

To analyze the digitalized images of each specimen using two different scanning devices — CCD camera

and FS, de- and remineralized areas were selected at 10 different sites of 5-μm width and 100−150 μm in depth from the surface. Adobe Photoshop 7.0 (Adobe Systems Incorporated, USA) was used for this procedure. As shown in Fig. 3, the digitalized microradiographic image of the lesion was oriented such that the outer lesion surface was displayed vertically on the left hand side at a given distance from the left margin of the image frame. Average gray values obtained by the two scanning devices (FS and CCD camera) were measured using NIH Image Ver 1.63 for Macintosh (developed at the US National Institutes of Health).

3. Converting gray values to corresponding aluminum thickness (t_{al}) values

For an equivalent thickness of the aluminum step wedge, the measured gray value of each site was converted into the corresponding t_{Al} value in μm. This was done using the 4th degree polynomial curve derived from the aluminum step wedge. Correlation coefficients were r^2=0.997 to 0.999, which were similar to those of previous studies[7,10].

4. Determining V(x) (Vol%) and mineral content profile from t_{Al} values

The mineral content profile was determined using the Angmar's formula[6] as follows:

$$V(x) = 100(A_n(x)/t\text{-}\mu_0)/(\mu_m\text{-}\mu_0)\ (Vol\%)$$

where t_s = measured thickness of specimen, μ_m = linear X-ray attenuation coefficient of mineral, μ_0 = linear X-ray attenuation coefficient of organic material with water, $A_n(x)$ = X-ray absorbance of tooth slice at point (x). For $A_n(x) = \mu_{Al} \times t_{Al}$, μ_{Al} = linear X-ray attenuation coefficient of aluminum. V(x) values at the inner sound enamel were measured and denoted as (Vs).

Next, V(x) values of enamel lesion were adjusted by a linear fit of two sets of coordinates. Line fit was computed using the averaged Vol% values at a blank area corresponding to 0% mineral and the averaged Vol% values at the inner sound enamel corresponding to 90% mineral content[5,6].

5. Calculating the following parameters from mineral content profile: mineral loss value (ΔZ), mineral lesion depth (Ld), surface zone depth (SZd), lesion body depth (LBd), outer surface zone (OSZ), inner lesion body (ILB), and sandwiched area (SA) between OSA and ILB

In the present study, the software was developed using Visual Basic for Applications combined with Microsoft Excel 2004 for Macintosh (Microsoft Incorporated, USA), as shown in Fig. 4. Differential analysis[16] was used to detect the minimum gradient point of mineral content profile (Fig. 5), which was essential for the calculations of SZd and LBd[13]. For SZd, it was defined as the depth to the maximum

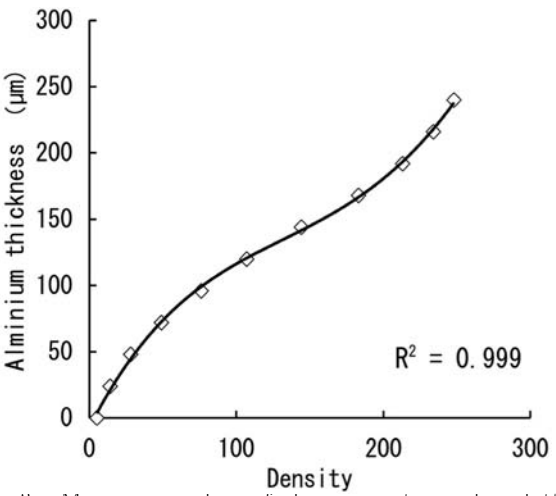


Fig. 2 Mean gray values of aluminum step wedge plotted against the thickness of aluminum wedge. The 4th degree polynomial curve of gray values *versus* aluminum thickness was fitted by least square method (r=0.999) using the LINEST function of Microsoft Excel.

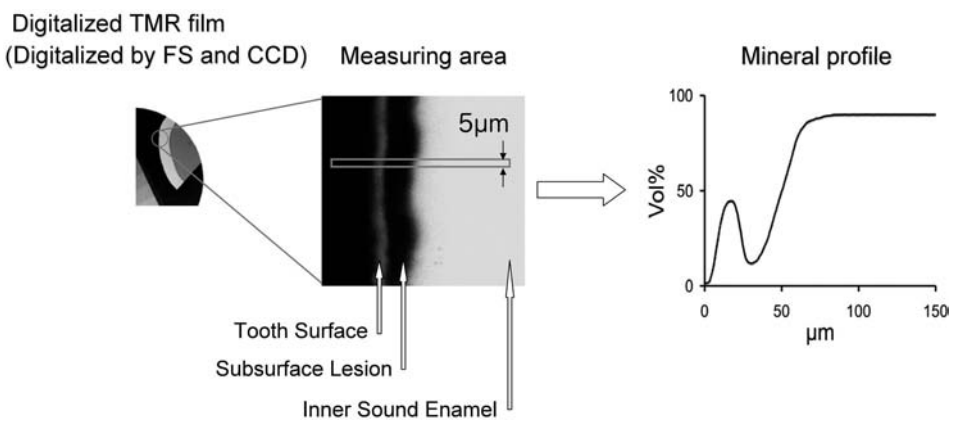
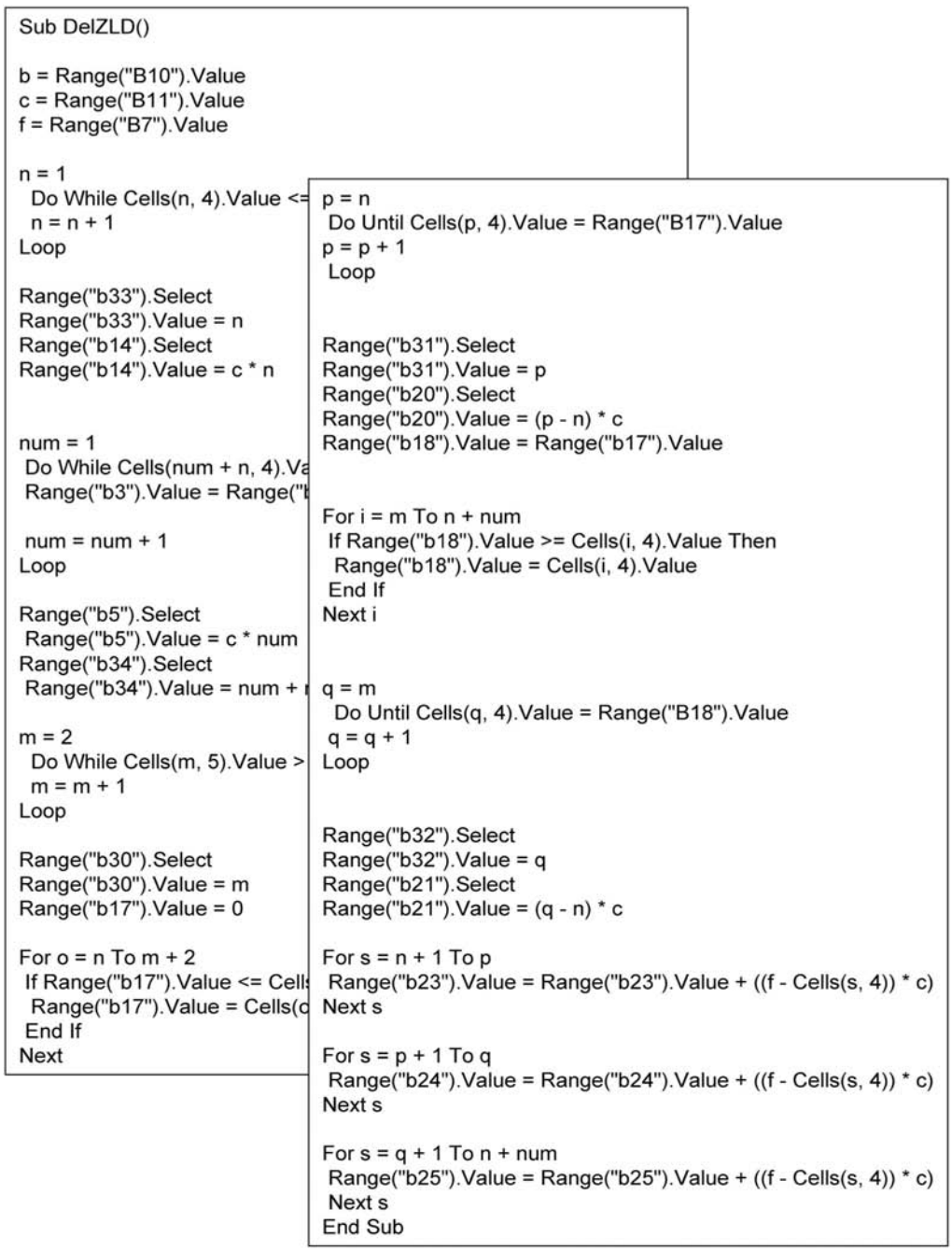


Fig. 3 Analysis area of a digitized radiographic image of a specimen by charge-coupled device camera with microscope (CCD camera). This figure shows digitalized area and corresponding mineral content profile.



```
Sub DelZLD()

b = Range("B10").Value
c = Range("B11").Value
f = Range("B7").Value

n = 1
  Do While Cells(n, 4).Value <=
  n = n + 1
Loop

Range("b33").Select
Range("b33").Value = n
Range("b14").Select
Range("b14").Value = c * n


num = 1
 Do While Cells(num + n, 4).Va
 Range("b3").Value = Range("

 num = num + 1
Loop

Range("b5").Select
 Range("b5").Value = c * num
Range("b34").Select
 Range("b34").Value = num +

m = 2
  Do While Cells(m, 5).Value >
  m = m + 1
Loop

Range("b30").Select
Range("b30").Value = m
Range("b17").Value = 0

For o = n To m + 2
 If Range("b17").Value <= Cells
  Range("b17").Value = Cells(o
 End If
Next
```

```
p = n
 Do Until Cells(p, 4).Value = Range("B17").Value
p = p + 1
 Loop


Range("b31").Select
Range("b31").Value = p
Range("b20").Select
Range("b20").Value = (p - n) * c
Range("b18").Value = Range("b17").Value


For i = m To n + num
 If Range("b18").Value >= Cells(i, 4).Value Then
  Range("b18").Value = Cells(i, 4).Value
 End If
Next i


q = m
  Do Until Cells(q, 4).Value = Range("B18").Value
 q = q + 1
Loop


Range("b32").Select
Range("b32").Value = q
Range("b21").Select
Range("b21").Value = (q - n) * c

For s = n + 1 To p
 Range("b23").Value = Range("b23").Value + ((f - Cells(s, 4)) * c)
Next s

For s = p + 1 To q
 Range("b24").Value = Range("b24").Value + ((f - Cells(s, 4)) * c)
Next s

For s = q + 1 To n + num
 Range("b25").Value = Range("b25").Value + ((f - Cells(s, 4)) * c)
 Next s
End Sub
```

Fig. 4 Newly developed program using Visual Basic for Applications combined with Microsoft Excel 2004 for Macintosh (Microsoft Incorporated, USA). This program could automatically calculate ΔZ, Ld, surface zone depth (SZd), lesion body depth (LBd), outer surface zone (OSZ), inner lesion body (ILB), and sandwiched area (SA) between OSA and ILB from mineral content profile.

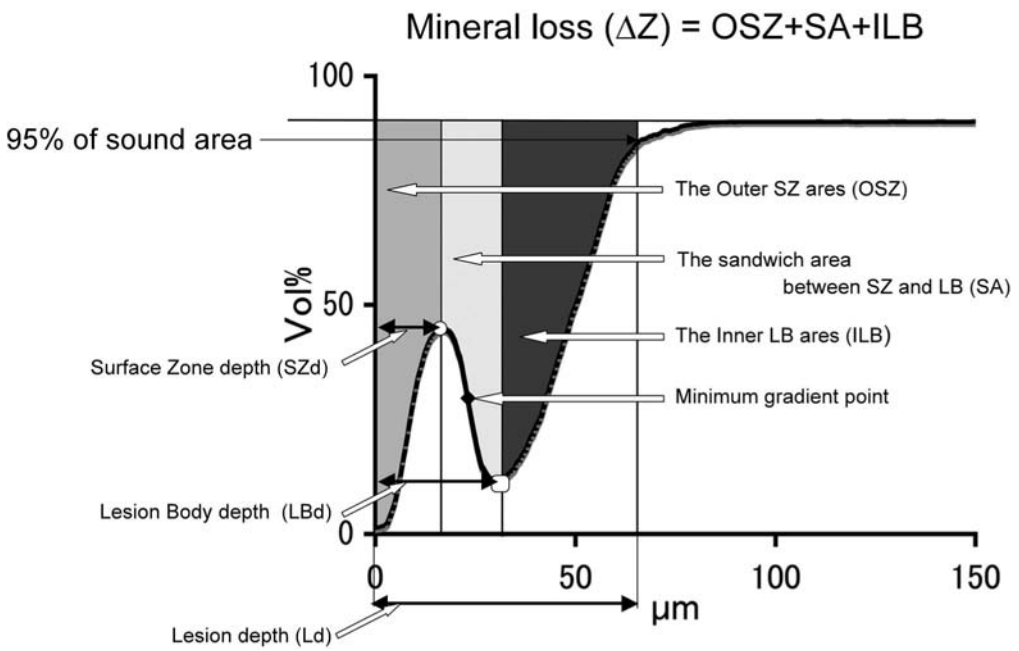


Fig. 5 Schematic drawing of an average mineral content profile showing total mineral loss in the lesion (ΔZ), lesion depth (Ld), surface zone depth (SZd), lesion body depth (LBd), outer surface zone (OSZ), inner lesion body (ILB), and sandwiched area (SA) between OSA and ILB.

point in the outer area of the minimum gradient point. For LBd, it was defined as the depth to the minimum point in the inner area of the minimum gradient point.

Based on these two depths, ΔZ was divided into three parameters — namely, OSZ, LBD, and SA, as shown in Fig. 5. Using the newly developed software, all the seven parameters of tooth enamel — namely, ΔZ, Ld, SZd, LBd, OSZ, ILB, and SA — were calculated automatically. Further, with this software, the outer surface was identified to be the point of 5 Vol%. Then, for each specimen, ΔZ (Vol%•μm), Ld (μm), SZd (μm), LBd (μm), and OSZ, ILB, SA (Vol%•m) of the 10 selected sites were calculated and compared between the two scanning devices of CCD camera and FS.

Statistical analysis

Data obtained were analyzed using a paired t-test at 5% level of significance (SPSS Ver. 10.0).

RESULTS

Figure 4 shows the newly developed software using Visual Basic for Applications combined with Microsoft Excel 2004 for Macintosh (Microsoft Incorporated, USA). This software was used to calculate ΔZ, Ld, SZd, LBd, OSZ, ILB, and SA of the de- and remineralized specimens (Fig. 5) of the present study. It did so by converting the gray

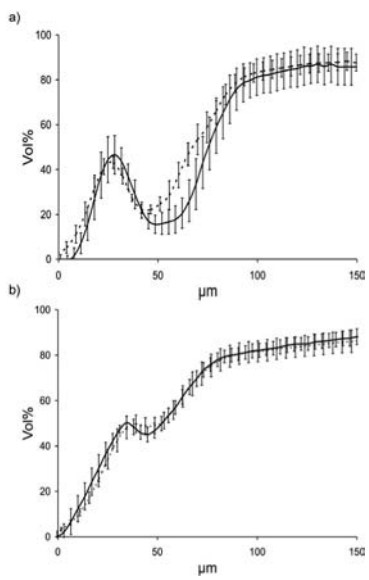


Fig. 6 (a) Demineralization and (b) remineralization mineral profiles of a subsurface lesion (averaged for 10 different sites) and their respective standard deviations (mean ± SD). Full line represents charge-coupled device camera with microscope (CCD camera) and dotted line represents digital film scanner (FS).

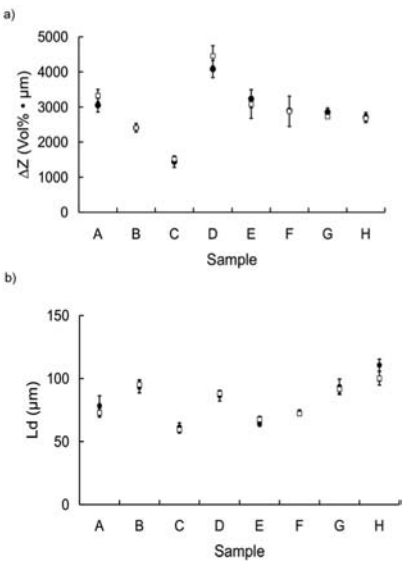


Fig. 7 (a) Average mineral loss values (**Δ**Z) and (b) average mineral lesion depths (Ld) with their standard deviations per scan site obtained in the eight different samples (A－H). Open squares represent CCD camera values and closed circles represent FS values. Statically paired t-test was performed between CCD camera and FS ($p<0.05$).

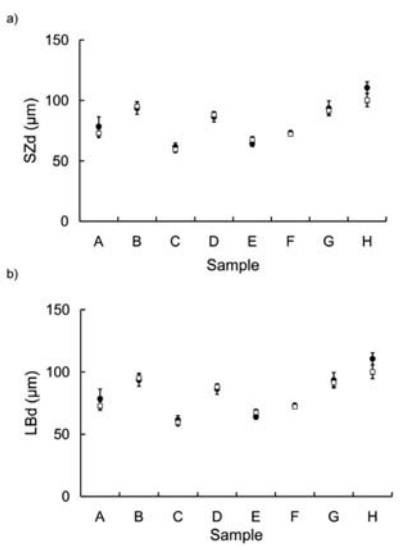


Fig. 8 (a) Average surface zone depths (SZd) and (b) average lesion body depths (LBd) with their standard deviations per scan site obtained with the eight different samples (A－H). Open squares represent CCD camera values and closed circles represent FS values. Statically paired t-test was performed between CCD camera and FS ($p<0.05$).

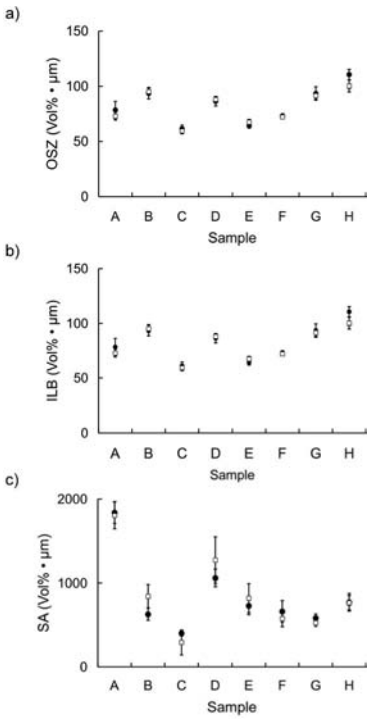


Fig. 9 (a) Average outer surface zone (OSZ) values; (b) average inner lesion body (ILB) values; and (c) average sandwiched area values between OSA and ILB (SA) with their standard deviations per scan site obtained in eight different samples (AH). Open squares represent CCD camera values and closed circles represent FS values. Statically paired t-test was performed between CCD camera and FS ($p<0.05$).

values — obtained by CCD camera or FS — to equivalent aluminum thicknesses. With the CCD camera, the Vol**%** of sound enamel (Vs) was 90.7±1.3, while that obtained by FS was 90.6±1.0. In other words, there were no significant differences in the Vs value as obtained by the two scanning devices.

Figure 3 reveals the TMR image as well as the digitalized profile by CCD camera at de- and remineralized areas of a sample. The former area was wider and darker than the latter area, and the demineralized area was clearly recovered in the digitalized profile after immersion in the remineralization fluid.

In Fig. 6, similar data were obtained by both FS and CCD for the remineralization pattern. However, the demineralization pattern seemed to differ between these two scanning devices at a depth range of 50－90 **μ**m. Notwithstanding the differences in demineralization pattern, no statistically significant differences ($p>0.05$) were observed at the 10 different sites among the eight samples (A－H) between the two scanning devices (CCD camera and FS) for these seven parameters — **Δ**Z, Ld, SZd, LBd, OSZ, ILB, and SA (Figs. 7, 8, and 9). A more detailed information

of the newly developed software will be available by contact to the corresponding author.

DISCUSSION

Quantitative contact microradiography is defined as transverse microradiography (TMR) where tooth slices are cut transversely to the labial surface of the tooth. According to a review by Arends and Bosch, TMR is the most widely used technique to evaluate tooth mineralization, despite some inherent disadvantages[4]. It has been reported that the accuracy of TMR for enamel was approximately 5 μm for Ld and approximately 200 for ΔZ (Vol%・μm), regardless of the digitalizing method[4]. In light of this finding[4], the TMR procedure was employed in the present study. To calculate the mineral content profile, TMR films were scanned. The long established method of scanning TMR films is to use a CCD camera[7–10]. Similarly, in the present study, this method was used to evaluate the de- and remineralization of tooth enamel in vitro as well as root caries lesions[7,10].

Alternatively, the digital film scanner (FS) is another available scanning device to trace TMR films. To the best of our knowledge, there is no published report that compares the data obtained by a CCD camera and those by a FS procedure. This is chiefly because FS became commercially available only recently (June 2003). With FS, its key merit lies in its wider scanning area (25060×37250 μm^2) — which is more than 2000 times larger than that of CCD camera (716×538 μm^2). Therefore, the specimen of interest and the standard aluminum wedge can be scanned together using a FS. At this juncture, it must also be mentioned that the resolution power of FS is lower than that of CCD camera. Resolution is 4.7 μm per pixel for the former, and 0.35 μm per pixel for the latter.

For an equivalent thickness of the aluminum step wedge, the measured gray value of each site was converted into the corresponding t_{Al} value in μm. This was done by using a 4th degree polynomial curve derived from the aluminum step wedge. The 4th degree polynomial method was used as described by De Josselin *et al.*[5], Lagerweij *et al.*[7], and Inaba *et al.*[10]. In these studies, the correlation coefficients were r^2=0.998, 0.997 to 0.999, which were similar to our results.

Our newly developed system comprised widely affordable and popularly used software applications — namely, NIH Image, Photoshop, and Microsoft Excel 2004. The foremost intent was that this new system could likewise be used freely and widely by other researchers.

The NIH Image Ver. 1.63 for Macintosh is a commonly used freeware to measure gray values from digital images, which were used for TMR analysis in a previous study[10]. Currently, there are three freeware programs available for Windows, Mac OS X (Scion Images, Scion Corporation, USA), and Linux ×86 (Image J, developed at the US National Institutes of Health). As for Adobe Photoshop, it is an image processing software of which the key advantage is that the images obtained can be layered. Therefore, the same area of different specimens could be compared by layering the TMR images. Last but not least, Microsoft Excel 2004 for Macintosh (Microsoft Incorporated, USA) is the most useful spreadsheet software, which contains a data calculation program using Visual Basic for Applications, as shown in Fig. 4.

Our software, when applied to the present study, was able to automatically calculate several parameters derived from the mineral content profile — namely, mineral loss value (ΔZ), mineral lesion depth (Ld), surface zone depth (SZd), lesion body depth (LBd), outer surface zone (OSZ), inner lesion body (ILB), and the sandwiched area (SA) between OSA and ILB, as shown in Fig. 5. In terms of software design concept, it was essentially based on the papers of Angmar *et al.*[6] and De Josselin *et al.*[5] as well as leveraging on the edge detection technique of the digital image processing method[16].

In a previous study by De Josselin *et al.*[5], TMRW software (Inspektor Research Systems BV, Amsterdam, the Netherlands)[17] was used to measure tooth mineralization. In that study[5], the Vol% of sound enamel (Vs) was 90.2±0.9, which was in good agreement with the values of 90.7±1.3 by CCD camera and 90.6±1.0 by FS procedure in this study. However, in a study by Angmar *et al.*, Vs was 86.2 Vol%[6]. The discrepancy between Angmar's value and our value might be caused by differences in tooth slice thickness, biological variation, and TMR method[5]. Individually or taken together, these differences could cause errors during the calculation of parameters such as ΔZ and Ld. With due consideration to these error possibilities, the Vs values in this study were adjusted by a linear fit of two sets of coordinates.

As seen in Figs. 7, 8, and 9, the data of ΔZ, Ld, SZd, and LBd by CCD camera or FS after de- or remineralization of the tooth specimens were in good agreement. The R value (R=ΔZ/Ld in Vol%), which was a parameter indicating the amount of mineral loss and the relationship between ΔZ and Ld, was 26.9±6.0 by CCD camera and 26.8±5.4 by FS in this study. These results corresponded fairly well with the values obtained in previous studies, ranging from 22±7 to 33±8[18–20]. As for the other three parameters — namely, OSZ, SA, and ILB — which might be useful for analyzing the progress of tooth de- and remineralization in detail, similar trends in data

agreement between CCD camera and FS were once again observed.

CONCLUSION

Based on the results obtained in the present study, it was adequately demonstrated that our newly developed software bore two distinctive merits of convenience and reliableness for tooth enamel mineralization analysis.

The results obtained herein were compared between two commonly used scanning devices of CCD camera and FS, as well as against the previous works of Angmar *et al.*[6] and De Josselin *et al.*[5].

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-aid for Scientific Research by the Japan Society for the Promotion of Science, Scientific Research, (C) (No. 13671992), and by a Grant-in-aid for Hokkaido University from the Clark Incorporated Foundation.

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