Received: 13 July 2020 | Revised: 19 August 2020 | Accepted: 21 August 2020

DOI: 10.1002/ajpa.24143

TECHNICAL NOTE

American Journal of PHYSICAL ANTHROPOLOGY | WILEY

An image analysis protocol for the quantification of interglobular dentine in anthropological tooth sections

Anne Marie E. Snoddy[1] | Justyna J. Miszkiewicz[2] | Carolina Loch[3] | Monica Tromp[1,4] | Hallie R. Buckley[1]

[1]Department of Anatomy, University of Otago, Dunedin, New Zealand

[2]School of Archaeology and Anthropology, Australian National University, College of Arts and Sciences, Canberra, Australia

[3]Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand

[4]Southern Pacific Archaeological Research, Archaeology Programme, School of Social Sciences, University of Otago, Dunedin, New Zealand

Correspondence
Anne Marie E. Snoddy, Department of Anatomy, University of Otago, 270 Great King Street, Dunedin, Otago 9016, New Zealand.
Email: annie.sohler@otago.ac.nz

Funding information
Marsden Fund, Grant/Award Number: 18-UOO-028

Abstract

The histological identification of interglobular dentine (IGD) in archeological human remains with macroscopic evidence of rickets has opened a promising new avenue for the investigation of metabolic disease in the past. Recent paleopathological studies have shown that histological analysis of archeological human teeth may allow the identification of periods of vitamin D deficiency occurring within very narrow developmental windows, yielding new information on the seasonality or even maternal-fetal transmission of this disease. However, currently available techniques for recording IGD rely on subjective scoring systems or visual estimations, potentially leaving them open to inter and intra-observer error and rendering comparisons of datasets difficult. Here we describe a new imaging protocol that utilizes open access software and may yield more objective and quantitative data on the amount of IGD present within a dentinal region of interest. We demonstrate that grayscale histograms in FIJI®/ImageJ® might be used to provide less subjective estimates of the percentage of a region of interest affected by IGD. Application of this technique may enable more accurate comparison of datasets between researchers.

KEYWORDS

dental histology, ImageJ®, paleopathology, vitamin D deficiency

1 | INTRODUCTION

Interglobular dentine (IGD) is a developmental defect in teeth, comprised of arc-shaped regions of poorly mineralized dentine bordered by unfused calcospherites (Nanci, 2018). Animal experimental studies have shown that IGD can coincide with odontoblast retraction in the dentine tubules as part of normal dental development (e.g., Kagayama, Zhu, Sasano, Sato, & Mayanagi, 1997; Tsuchiya, Sasano, Kagayama, & Watanabe, 2002). Research using modern human teeth has also noted that IGD is distributed unevenly across the root and the crown, because of regional tooth differences in the retraction of odontoblast processes (Jayawardena, Nandasena, Abeywardena, & Nanayakkara, 2009). However, a clinical association has been demonstrated between a greater presence of IGD and both inherited and acquired mineralization disorders such as familial hypophosphatemic and nutritional rickets (Seow, Romaniuk, & Sclavos, 1989; Shellis, 1983; Tracy, Steen, Steiner, & Buist, 1971). IGD has also been histologically observed in paleopathological examples of individuals with macroscopic evidence of rickets (D'Ortenzio et al., 2016; Veselka et al., 2019). In recent years, histological analysis of IGD from archeological individuals has received increased attention due to its potential to provide evidence of vitamin D deficiency (VDD) occurring during discrete developmental periods that are too subtle or brief to manifest macroscopically (Brickley, Kahlon, & D'Ortenzio, 2019; Colombo et al., 2018; D'Ortenzio et al., 2016).

Histological analysis of teeth is rapidly gaining traction in the paleopathological literature as it can provide high-resolution, chronologically precise information on defects associated with physiological stress during development. This destructive method involves creating thin sections (~100 μm) of plastic embedded teeth and viewing the

internal structures, such as enamel prisms and dentine tubules, with brightfield or polarized light microscopy (see Aris, 2020 for a review). To date, both clinical and anthropological methods of estimating the severity of IGD formation have involved either subjective scoring systems (Isokawa, Kosakai, & Kajiyama, 1963; Mellanby, 1928) or visual estimation of the percentage of dentine tissue affected within a region of interest (ROI) (D'Ortenzio et al., 2016; Veselka et al., 2019). Although visual estimation methods are an improvement on scoring systems, they are still subjective and open to high degrees of both inter and intra-observer error, because they rely on an approximate estimation of the extent to which IGD occurs in a given tooth area. In particular, methods that estimate percentage of IGD can be ambiguous as it is difficult to identify whether they concern the proportion of dentinal tissue containing IGD only or the cumulative value of the voids created by unmineralized calcospherites (e.g., D'Ortenzio et al., 2016, pp. 157–158). Although D'Ortenzio and colleagues have accounted for some observational variation by creating broad severity grades (e.g., Grade 2 = 25–50%), we believe a more quantitative approach would be beneficial, especially when comparing datasets. These recent histological reports examining IGD in archeological samples are pioneering (e.g., Brickley et al., 2019; D'Ortenzio et al., 2016; Veselka et al., 2019) and have contributed greatly to the study of metabolic bone disease in past populations. However, as with all developing methodologies, it is important to work toward more objective techniques that reduce the potential for observational error. The aim of this technical note is to outline an image analysis protocol for quantifying IGD which reduces the potential for inter and intra-observer error. We use the open-access software FIJI®, which is a version of ImageJ® (v. 1.52) that includes preinstalled plugins used in scientific image analysis (Schindelin et al., 2012).

2 | THE PROTOCOL: GRAYSCALE HISTOGRAMS IN FIJI®

The following is a protocol summary for calculating the percentage of a ROI within primary dentine which is composed of IGD defects. The full protocol, with corresponding screenshots of each step, is provided in the Supporting Information. To begin, download FIJI® and import your slide scan into the FIJI/ImageJ® (v. 1.52) window. Convert the image to grayscale (8 bit). Use the line segment tool and trace scale bar in scanned image to set scale in mm. Add grid overlay from the plugins tab; this will need to be downloaded separately and installed in FIJI (see Supporting Information). Set the grid boxes to 0.5 mm^2. Use the rectangle tool to select the area within a grid box of interest. Cut this area out into a separate file. Threshold this new image to reduce background noise (variation in contrast unrelated to IDG) ensuring that the IGD in new image matches IGD from the original image as closely as possible (see Section 3). Regions of true IGD should appear as purely black voids. Quantify IGD by copying pixel data into an Excel spreadsheet, and calculating percentage of IGD:

(#black pixels/ # total pixels[#white pixels + # black pixels]) × 100 = % ROI with IGD

The core technical principle of our protocol is the generation of grayscale histograms from dental histology images. Grayscale histograms are often used in research that deals with estimation of skeletal tissue density or mineralization changes (Dias, Cook, & Mirhosseini, 2011; Palacio-Mancheno, Larriera, Doty, Cardoso, & Fritton, 2014; Waung et al., 2014). This involves analyzing the distribution of gray value pixels within an image or a specific ROI. IGD can be visualized microscopically in unstained thin sections as darker, irregular voids within globular dentine areas (Nanci, 2018; Figure 1). By converting a high resolution slide-scan image to 8-bit grayscale and increasing the contrast, the IGD voids become enhanced as regions of black pixels against white background (Figure 2). The grid overlay plugin (Rasband, 2016) can be used to divide the image into identically sized areas from which a single ROI can be isolated, ensuring greater consistency across multiple samples (provided the same scale is used each time). Running the histogram analysis function in the Fiji® software will produce a total number of black and white pixels on the grayscale. These can then be used to calculate the "true" percent of IGD in the ROI. The resulting percentages become quantitative data rather than estimated percentage categories. The application of our protocol to an image shown in D'Ortenzio et al. (2016, p. 157, figure 4c therein) results in an IGD count of only ~6.5%, while the authors give this a visual estimate of 25–50% coverage ("Grade 2"). This discrepancy may in part be due to differences in the magnification and grid size between the two methods. However, it is clear that the severity categories provided in previous publications might have over-estimated IGD presence.

3 | METHODOLOGICAL CONSIDERATIONS

In order for this protocol to be effective, the following issues need to be considered:

- Defects in the histological slide, such as air bubbles and artifacts from mounting or embedding, will need to be erased from the scanned image prior to running the histogram; otherwise, they risk artificially enhancing the IGD count. This can be achieved by using the color picker tool to choose a neutral background color to match the unaffected dentine and then coloring in the bubble or artifact with the paintbrush tool.
- Thresholding will not result in a perfect representation of interglobular voids and there is an element of subjectivity inherent in this process. This can be seen in Figure 2, where the adjusted image has a very slightly underestimated IGD compared to the original. However, so long as care is taken with visual matching the resulting histogram will provide a more objective measure of IGD than visual estimation alone.
- Analysis of regions of dentine with naturally high color contrast, such as those containing dead tracts or reparative dentine, should be avoided as these will influence the grayscale histogram.
- The grid from which ROIs are derived must be set to a standard across all images in the dataset. For our analyses here, we have

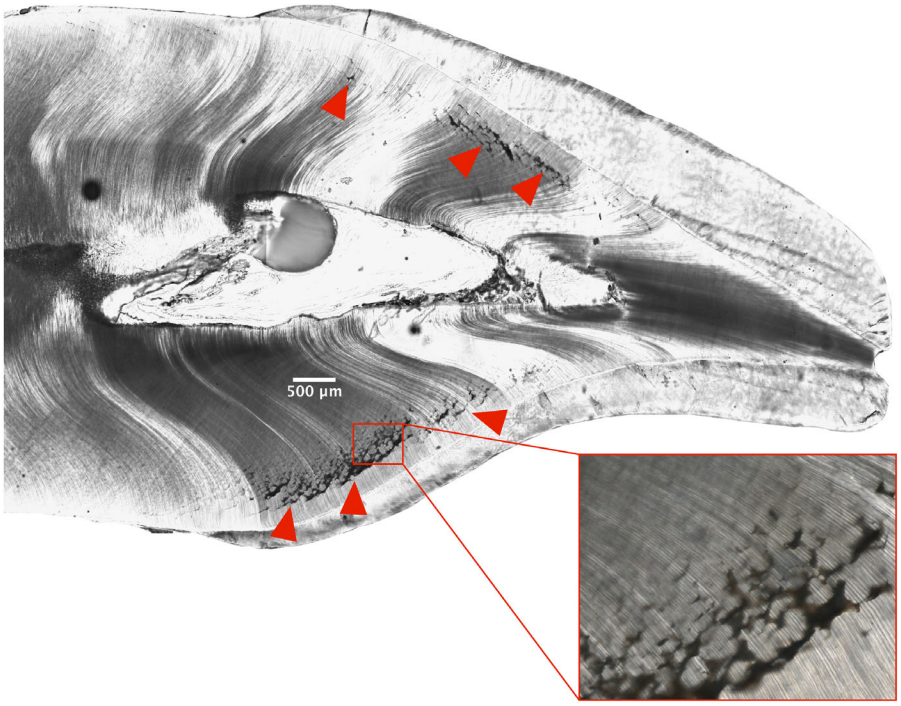

FIGURE 1 Labio-lingual thin section of lateral mandibular incisor. Red arrows indicate areas of IGD. Inset box shows a close-up of interglobular voids within dentine. IGD, interglobular dentine

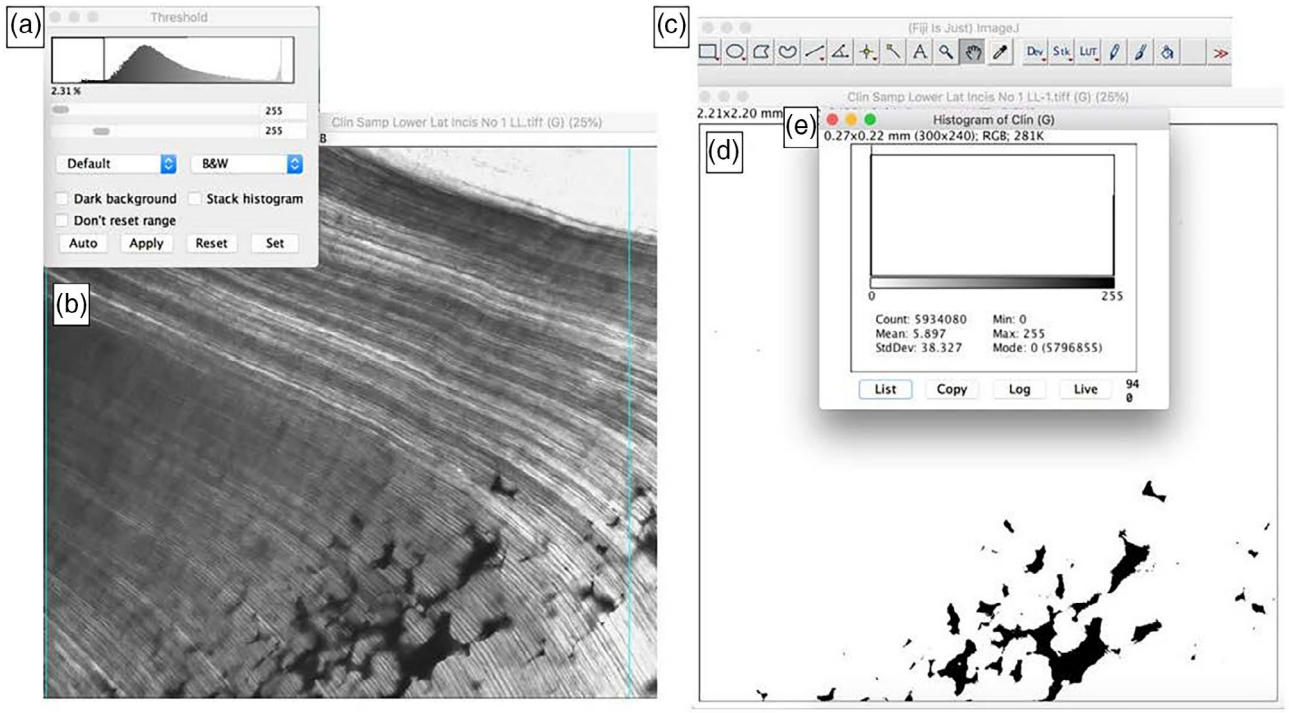

FIGURE 2 Grayscale histogram in FIJI®/ImageJ®(c) revealing IGD as regions of black pixels (d). The histogram window (e) shows the grayscale distribution (0–255) with 0 indicating white pixels, and 255 indicating black pixels. On the left, a pre-contrasted, but grayscaled image (b) can be seen. IGD, interglobular dentine

chosen a grid of 0.5mm^2. This is, to some degree arbitrary but in our experience larger boxes will make it more difficult to identify IGD voids at high enough resolution for the protocol to be effective.

- Histological sections compared must be of a uniform thickness (Bromage & Werning, 2013). Because IGD defects can superimpose when viewed two-dimensionally, variation in section thickness may influence the amount of dentine exposed. For example,

portions of IGD located closer to the bottom surface of the thin section will appear blurred and distanced if the image was taken with a microscope objective focused on the most superior/superficial surface of the section.

- Where possible, slide imaging should be undertaken using a "z-stacking" option, so that multiple images in the z-plane are combined and flattened. This increases the depth of field of the image and will help mitigate some of the inevitable variation in specimen thickness that occurs even with the use of a hand-held precision grinder.

4 | CONCLUSIONS

We have reported a quantitative method of recording IGD in archeological samples of human teeth. This protocol uses freely available software and provides a means to reduce inter and intra-observer error that will facilitate comparison of datasets between research groups going forward. More work is needed in order to formally test and compare inter and intra-observer error rates between this protocol and other methods of recording IGD, and to establish an evidence-based threshold for the amount of IGD that can be considered pathological. Additional research is also needed to determine whether factors such as variation in section direction, section thickness, or the region of tooth observed (e.g., crown vs. root) influence the amount of IGD recorded. However, this protocol offers a first step for methodological improvement in this research area.

ACKNOWLEDGMENTS

The authors would like to thank the anonymous reviewers for their comments. This work was funded by a Marsden Fund Grant (18-UOO-028) awarded to Hallie R. Buckley.

CONFLICT OF INTEREST

The authors declare no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Anne Marie Sohler-Snoddy: Conceptualization; formal analysis; methodology; writing-original draft; writing-review and editing. **Justyna Miszkiewicz:** Conceptualization; methodology; writing-review and editing. **Carolina Loch:** Methodology; writing-review and editing. **Monica Tromp:** Methodology; software; writing-review and editing. **Hallie Buckley:** Project administration; resources; supervision; writing-review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

ORCID

Anne Marie E. Snoddy https://orcid.org/0000-0002-0516-785X
Justyna J. Miszkiewicz https://orcid.org/0000-0002-9769-2706

REFERENCES

Aris, C. (2020). The histological paradox: Methodology and efficacy of dental sectioning. *Papers from the Institute of Archaeology*, *29*(1), 1–16. http://doi.org/10.5334/pia-548

Brickley, M. B., Kahlon, B., & D'Ortenzio, L. (2019). Using teeth as tools: Investigating the mother–infant dyad and developmental origins of health and disease hypothesis using vitamin D deficiency. *American Journal of Physical Anthropology*, *25*(3), 95–12. http://doi.org/10.1002/ajpa.23947

Bromage, T., & Werning, S. (2013). Image standardization in paleohistology. In K. Padian & E. T. Lamm (Eds.), *Bone histology of fossil tetrapods: Advancing methods, analysis, and interpretation* (pp. 161–176). Berkeley, California: University of California Press.

Colombo, A., D'Ortenzio, L., Bertrand, B., Coqueugniot, H., Knüsel, C. J., Kahlon, B., & Brickley, M. (2018). Micro-computed tomography of teeth as an alternative way to detect and analyse vitamin D deficiency. *Journal of Archaeological Science: Reports*, *23*, 390–395. http://doi.org/10.1016/j.jasrep.2018.11.006

Dias, G. J., Cook, R. B., & Mirhosseini, M. (2011). Influence of food consistency on growth and morphology of the mandibular condyle. *Clinical Anatomy*, *24*(5), 590–598. https://doi.org/10.1002/ca.21122

D'Ortenzio, L., Ribot, I., Raguin, E., Schattmann, A., Bertrand, B., Kahlon, B., & Brickley, M. (2016). The rachitic tooth: A histological examination. *Journal of Archaeological Science*, *72*, 152–163. http://doi.org/10.1016/j.jas.2016.06.006

Isokawa, S., Kosakai, T., & Kajiyama, S. (1963). Interglobular denin in the deciduous tooth. *Journal of Dental Research*, *42*, 831–834. https://doi.org/10.1177/00220345630420031301

Jayawardena, C., Nandasena, T., Abeywardena, A., & Nanayakkara, D. (2009). Regional distribution of interglobular dentine in human teeth. *Archives of Oral Biology*, *54*(11), 1016–1021. https://doi.org/10.1016/j.archoralbio.2009.09.001

Kagayama, M., Zhu, J. X., Sasano, Y., Sato, H., & Mayanagi, H. (1997). Development of interglobular dentine in rat molars and its relation to maturation of enamel. *Anatomy and Embryology*, *196*(6), 477–483. https://doi.org/10.1007/s004290050115

Mellanby, M. T. (1928). The influence of diet on the structure of teeth. *Physiological Reviews*, *8*(4), 545–577. https://doi.org/10.1152/physrev.1928.8.4.545

Tsuchiya, M., Sasano, Y., Kagayama, M., & Watanabe, M. (2002). The extent of odontoblast processes in the dentin is distinct between cusp and cervical regions during development and aging. *Archives of Histology and Cytology*, *65*(2), 179–188. https://doi.org/10.1679/aohc.65.179

Nanci, A. (2018). *Ten Cate's oral histology: Development, structure, and function*. St. Louis, MO: Elsevier.

Palacio-Mancheno, P. E., Larriera, A. I., Doty, S. B., Cardoso, L., & Fritton, S. P. (2014). 3D assessment of cortical bone porosity and tissue mineral density using high-resolution μCT: Effects of resolution and threshold method. *Journal of Bone and Mineral Research*, *29*(1), 142–150. https://doi.org/10.1002/jbmr.2012

Rasband, W. (2016). Grid overlay. Retrieved from https://imagej.nih.gov/ij/plugins/graphic-overlay.html.

Seow, W. K., Romaniuk, K., & Sclavos, S. (1989). Micromorphologic features of dentin in vitamin D-resistant rickets: Correlation with clinical grading of severity. *Pediatric Dentistry*, *11*(3), 203–208.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzch, T., … Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. http://doi.org/10.1038/nmeth.1985

Shellis, R. P. (1983). Structural organization of calcospherites in normal and rachitic human dentine. *Archives of Oral Biology*, *28*(1), 85–95. http://doi.org/10.1016/0003-9969(83)90030-4

Tracy, W. E., Steen, J. C., Steiner, J. E., & Buist, N. R. M. (1971). Analysis of dentine pathogenesis in vitamin D-resistant rickets. *Oral Surgery, Oral*

Medicine, and Oral Pathology, *32*(1), 38–44. http://doi.org/10.1016/0030-4220(71)90248-9

Veselka, B., Brickley, M. B., D'Ortenzio, L., Kahlon, B., Hoogland, M. L. P., & Waters Rist, A. L. (2019). Micro-CT assessment of dental mineralization defects indicative of vitamin D deficiency in two 17th–19th century Dutch communities. *American Journal of Physical Anthropology*, *169*(1), 122–131. http://doi.org/10.1002/ajpa.23819

Waung, J. A., Maynard, S. A., Gopal, S., Gogakos, A., Logan, J. G., Williams, G. R., & Bassett, J. H. D. (2014). Quantitative X-ray microradiography for high-throughput phenotyping of osteoarthritis in mice. *Osteoarthritis and Cartilage*, *22*(10), 1396–1400. https://doi.org/10.1016/j.joca.2014.04.015

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Snoddy AME, Miszkiewicz JJ, Loch C, Tromp M, Buckley HR. An image analysis protocol for the quantification of interglobular dentine in anthropological tooth sections. *Am J Phys Anthropol*. 2021;174:144–148. https://doi.org/10.1002/ajpa.24143