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News and Views

Nondestructive imaging of hominoid dental microstructure using phase contrast X-ray synchrotron microtomography

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Anthropological significance of dental microstructure

Numerous studies have examined aspects of tooth development to elucidate evolutionary relationships among apes and humans, as well as processes that led to the prolonged life history of modern humans (reviewed in Smith and Tompkins, 1995; Smith, 2004; Dean, 2006). Many of these studies utilize incremental dental development, or the biological rhythms recorded in teeth (reviewed in FitzGerald, 1998; Dean, 2006; Smith, 2006), to characterize the rate and duration of tooth growth. Histological approaches permit more accurate estimates of crown formation time than radiographic methods (e.g., Beynon et al., 1998a), and may provide highly accurate estimates of age at death in developing dentitions (e.g., Antoine, 2000; Smith et al., 2006a). These techniques have led to new interpretations of hominin developmental processes (e.g., Dean et al., 2001), information about the evolution of Miocene hominoid life history (e.g., Beynon et al., 1998b; Kelley and Smith, 2003), and variation in living hominoid dental development (e.g., Reid and Dean, 2000; Schwartz et al., 2001; Reid and Dean, 2006; Schwartz et al., 2006; Smith et al., 2007a,b).

During enamel formation, hydroxyapatite crystallites are bound together in long, thin enamel prisms, representing accretionary products of enamel-forming cells. Enamel prisms preserve a record of daily secretion, represented by circadian features termed cross-striations and/or laminations (Bromage, 1991; Smith, 2006; Tafforeau et al., 2007). The successive positions of the forming front are also regularly preserved as long-period features known as Retzius lines, which meet the surface of the lateral enamel and form perikymata (circumferential external rings). In order to accurately assess tooth crown formation, previous studies of intact teeth have employed physical sectioning of tooth crowns to visualize the key relationship between cross-striations and Retzius lines: the periodicity of long-period lines (i.e., number of days between successive Retzius lines), as well as the enamel secretion rate and cuspal enamel thickness. To date, data from only 18 sectioned hominin fossil teeth are published (Grine and Martin, 1988; Dean et al., 1993; Dean et al., 2001; Ward et al., 2001; Sasaki et al., 2002; Lacruz et al., 2006; Macchiarelli et al., 2006; Smith et al., in press), representing a paucity of directly observed hominin developmental data. While certain developmental features may be fairly consistent within a species and among teeth (e.g., daily secretion rate; Smith et al., 2007a), other features such as the periodicity of Retzius lines or cuspal enamel thickness may show a high degree of intraspecific variation (e.g., Schwartz et al., 2001; Reid and Dean, 2006; Smith et al., 2007a,b). This variability implies that assessments of formation time employing estimates of internal parameters, such as the Retzius line periodicity, may necessitate large confidence intervals.

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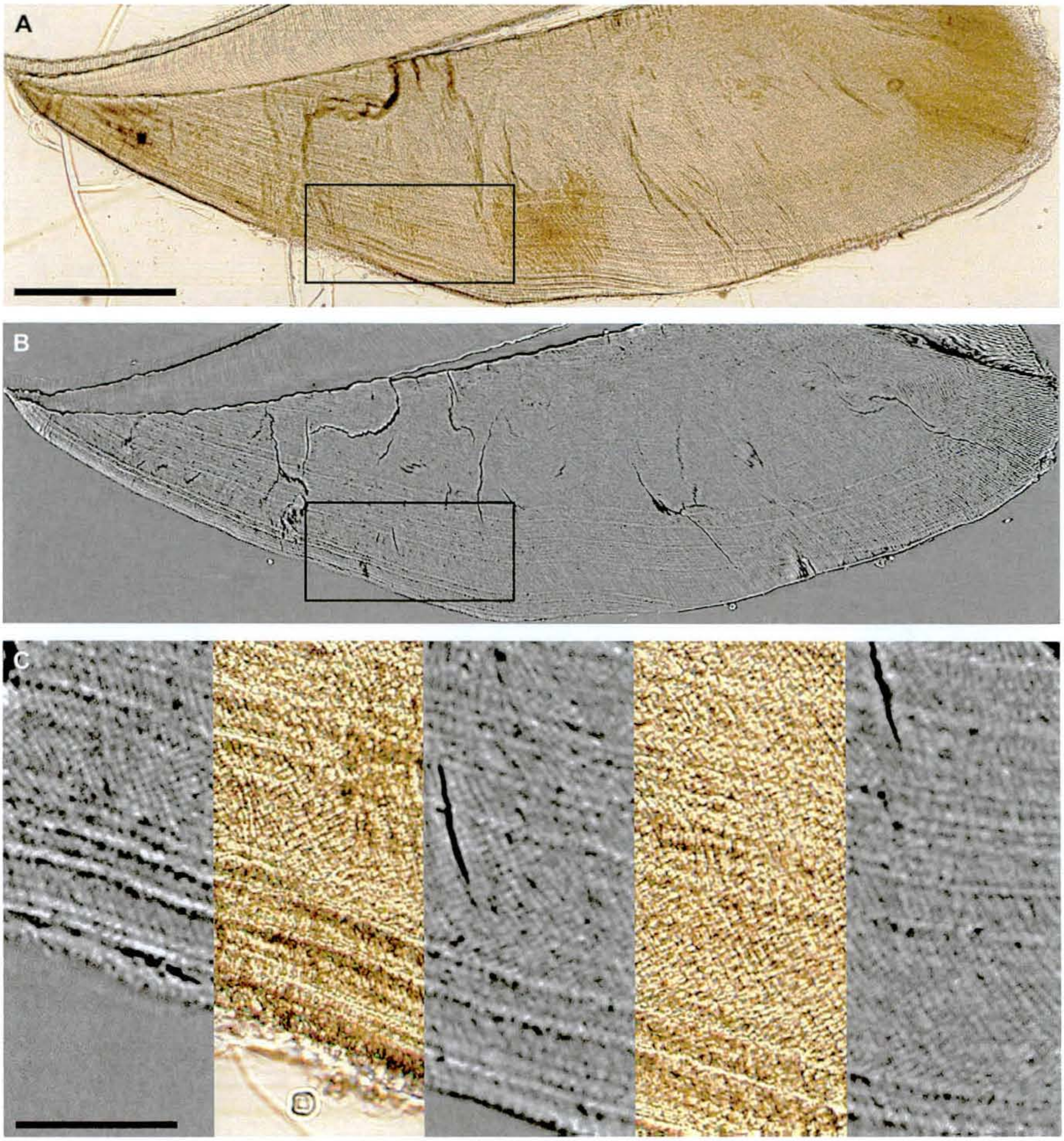

Fig. 1. Comparison between (A) 100-μm-thick histological section of a developing chimpanzee cusp imaged with light microscopy (after sectioning), and (B) the same virtual slice (3.5 μm thick) imaged with phase contrast SR-mCT (prior to sectioning). The virtual slice is an average of five successive 0.7-μm slices. (C) Enlargement of the subsurface enamel (black boxes in A and B) showing good correspondence of incremental features. Scale bars represent 500 μm in A and B, and 100 μm in C. See Smith et al. (2007b) for details of the histological slice preparation.

Synchrotron imaging

X-rays are electromagnetic radiation with wavelengths much shorter than visible light. They have been used for over a century to reveal information about the internal structure of objects, initially through radiographic projections (representing the differential absorption of the X-rays by the object), and more recently through the development of computed tomography. Tomography is the computerized reconstruction of cross-sectional slices using algorithms on radiographs collected during rotation. Stacks of these slices are used to generate three-dimensional representations of an object, allowing exploration of inaccessible morphology and/or quantification of three-dimensional object properties (e.g., surface area, volume, density).

A synchrotron is a system of particle accelerators that use electromagnetic fields to accelerate and guide charged particles (generally electrons) around a circular track in a storage ring. When the accelerated particles are deflected by a magnetic field, they emit synchrotron light. Synchrotron microtomography techniques represent a substantial improvement over conventional microtomography (reviewed in Tafforeau et al., 2006). One of the most important features of synchrotron radiation is the production of X-ray beams that may exhibit high spatial coherence (a physical property that relates to wave geometry), allowing phase contrast imaging. Phase contrast is caused by modification of the geometry of a planar wavefront due to differences in the composition or density of an object. Phase contrast synchrotron microtomography (SR-mCT) may be used to image structures that are invisible with standard microtomographic absorption techniques, as demonstrated on various fossil samples (illustrated in Tafforeau et al., 2006). In the case of submicron imaging of dental microstructures, it should be noted that, although SR-mCT is nondestructive, in some cases there is a temporary darkening (colored-center effect) during scanning. Illumination with ultraviolet light accelerates the restoration of the enamel to its original appearance.

Incremental features of enamel microstructure are ideal subjects for phase contrast SR-mCT imaging because they are a result of both chemical and structural variations of enamel prisms (Boyde, 1989). Tafforeau (2004) first applied this technique to a primate fossil by illustrating the enamel microstructure in a partially demineralized cusp tip of the Eocene anthropoid primate *Siamopithecus* (see Figure 8 in Tafforeau et al., 2006), and later quantified the degree of mineralization of a modern rhinoceros tooth germ (see Figures 8–10 in Tafforeau et al., 2007). Smith et al. (2007c) similarly illustrated Retzius line periodicity in an early *Homo sapiens* enamel fragment. While these initial applications demonstrate that phase contrast SR-mCT imaging represents a promising approach to characterize the microscopic structure of mineralized tissues, a number of questions remain about the limits of this new application. For example, it is unclear if this method produces results directly comparable to light microscope investigation of tooth microstructure, whether developmental features such as the neonatal line may be identified, whether incremental features may be imaged throughout complete, fully formed hominoid teeth, and finally if images are comparable between recent and fossilized teeth.

Proof of concept

In order to assess microstructure visualization using phase contrast SR-mCT, developing chimpanzee tooth germs were imaged prior to and after physical sectioning. The fidelity of "virtual histology" is apparent on the complete slice and at the prismatic level (Fig. 1); images may be aligned to within a single 0.7-μm pixel, and measurements of cross-striation spacing taken in the same area of the physical and virtual section differ by less than 1%. Comparisons were also made between phase contrast SR-mCT and confocal microscopy, also yielding very similar information. However, unlike confocal microscopy, phase contrast SR-mCT is not limited to subsurface regions, and incremental features often appear more resolved due to the high isotropic resolution. Using phase contrast SR-mCT, it is possible to reveal structures smaller than the voxel size (0.7 μm in the present study or 0.28 μm in Tafforeau et al. [2007]). Each tomographic slice is much thinner than a traditional histological section, and by averaging successive slices it is possible to generate thicker slices to selectively enhance specific incremental structures (e.g., see Figure 10 in Tafforeau et al., 2007). It is also possible to identify periods of developmental stress as with histological sections; the neonatal line was clearly seen in a developing human tooth germ (Fig. 2), representing a novel nondestructive

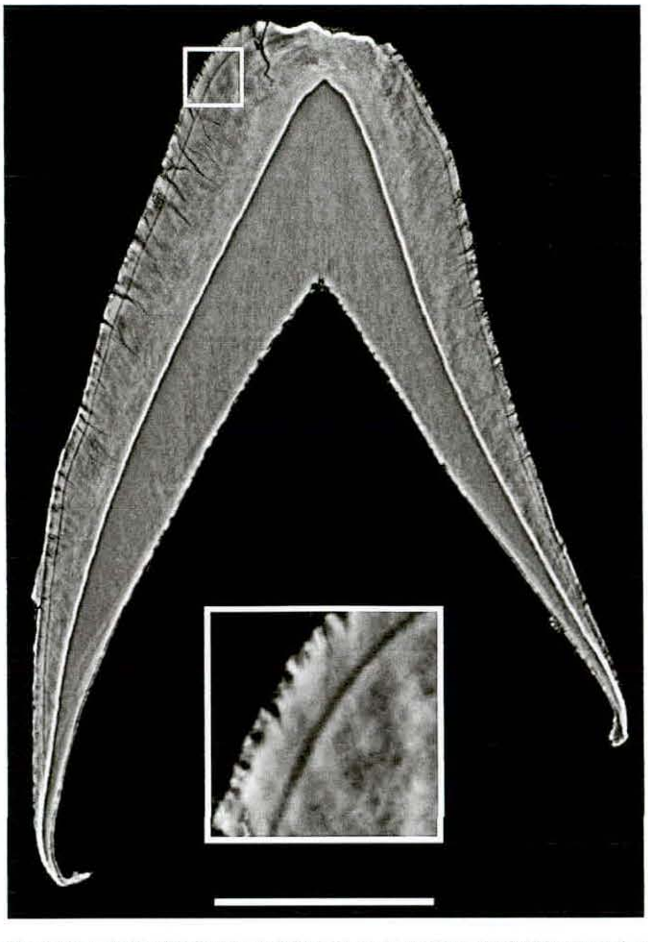

Fig. 2. Virtual slice (25.2 μm thick) through a modern human deciduous canine germ scanned with phase contrast SR-mCT using a voxel size of 5.03 μm. The dark line running parallel to the tooth surface is the neonatal line formed during birth (shown below in the box). This individual died shortly after birth, as evidenced by the lack of postnatal enamel and confirmed by records from the Institute of Anatomy, University of Bern. The scale bar is 1 mm.

approach to confirm postnatal survivorship and to relating developmental time to chronological age. Postnatal developmental stress may also be identified in fossil material (e.g., see Figure 2 in Smith et al., 2007c), which may allow teeth to be registered across the dentition, providing ages for crown initiation and completion (e.g., Schwartz et al., 2006).

Previously published studies of phase contrast SR-mCT were performed on small samples (less than 2 mm in width), which do not require high energy and local tomography (field of view smaller than the object). Recent developments demonstrate that this technique is effective for visualizing dental microstructure throughout the entire crown of whole teeth from recent and fossil hominoids (Fig. 3). Fossil material imaged with phase contrast SR-mCT often shows more clearly defined microstructure than fully calcified extant material, as is true for light microscopy (although mineralizing extant tooth germs are ideal for phase contrast: Figs. 1 and 3a; see also Tafforeau et al., 2007). Moreover, it is possible to image microstructure in hominin fossils more than two million years old (Fig. 4), potentially increasing the number of hominin taxa available for studies of incremental development.

Although it is possible to see dental microstructure on all samples imaged with phase contrast SR-mCT, the quality of results varies greatly depending on the sample. In some cases, it is impossible to assess Retzius line periodicity due to poor visibility of cross-striations or Retzius lines, which is also true with light and confocal microscopy, suggesting that this is due, in part, to intrinsic structural phenomena. The best results on fossil teeth generally derive from partially demineralized areas (e.g., see Figure 8 in Tafforeau et al., 2006; Figure 2 in Smith et al., 2007c), although this condition is not always necessary for effective imaging (Figs. 3b, 4).

Paleoanthropological applications

Of particular paleoanthropological significance is the nondestructive determination of long-period line periodicity using phase contrast SR-mCT (e.g., Fig. 4; also see Smith et al., 2007c). Particular emphasis has been placed on comparisons of external long-period features—perikymata—between Neandertals and modern humans (e.g., Ramirez Rozzi and Bermudez de Castro, 2004; Guatelli-Steinberg et al., 2005). However, without sectioning a tooth from an individual's dentition, comparisons of perikymata numbers are problematic, as the actual time represented by these features is unknown. By revealing long-period line periodicity, phase contrast SR-mCT may help to resolve the debate over life-history differences between Neandertals and living humans, and it can be used to investigate dental development in some of the earliest hominin fossils recovered (e.g., *Sahelanthropus tchadensis*; Brunet et al.,

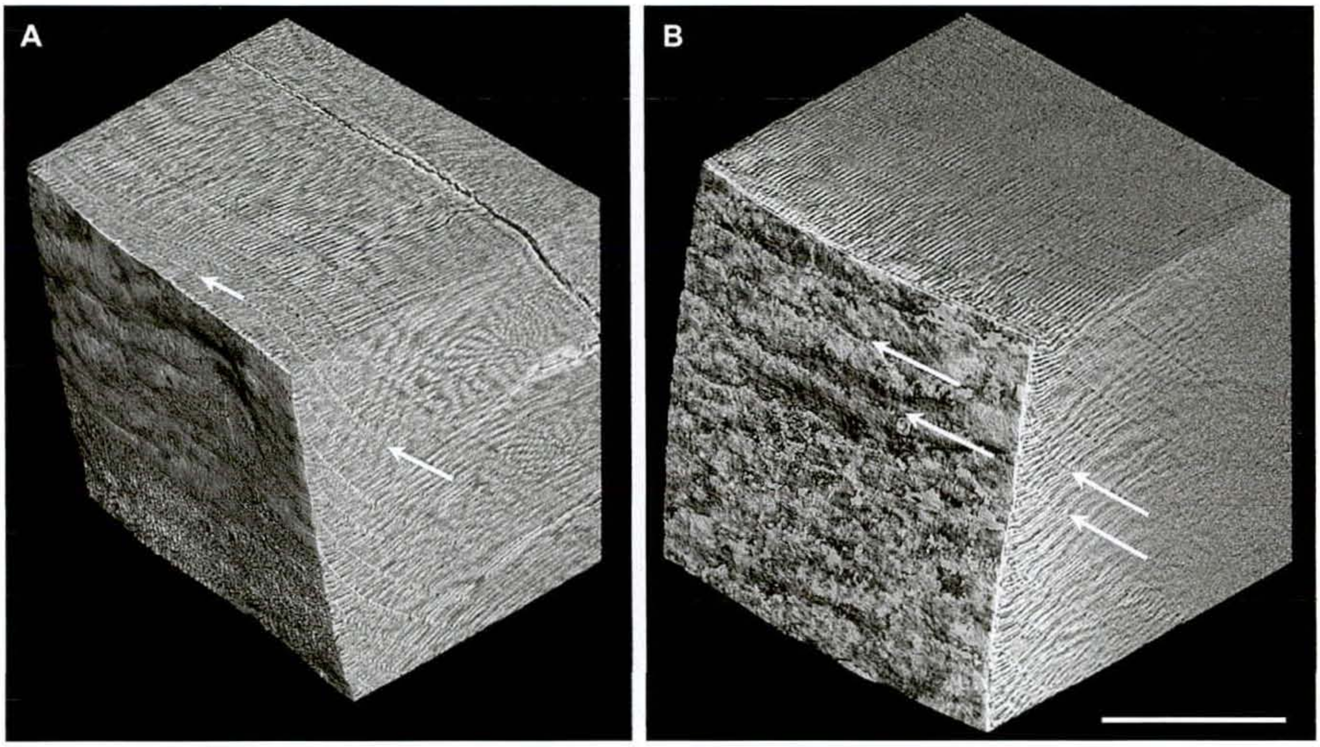

Fig. 3. Three-dimensional (3D) renderings of the lateral enamel in living (A) and fossil (B) orangutan molars. (A) Continuous Retzius line (white arrow) in tangential (superior face) and perpendicular orientations (right face). The specimen is a lower first molar germ that was refluxed and embedded in methylmethacrylate prior to imaging. (B) Retzius lines (white arrows) approaching the surface of the enamel (right face) and terminating on the surface of the enamel as perikymata (white arrows) (left face). This complete, fully formed crown derives from a collection of fossil *Pongo* recovered from Tubo Cave, south China (~70,000 years ago). Approximately seven to eight Retzius lines can be seen terminating as perikymata on the surface of each 3D block. Scale bar is 250 μm for both blocks.

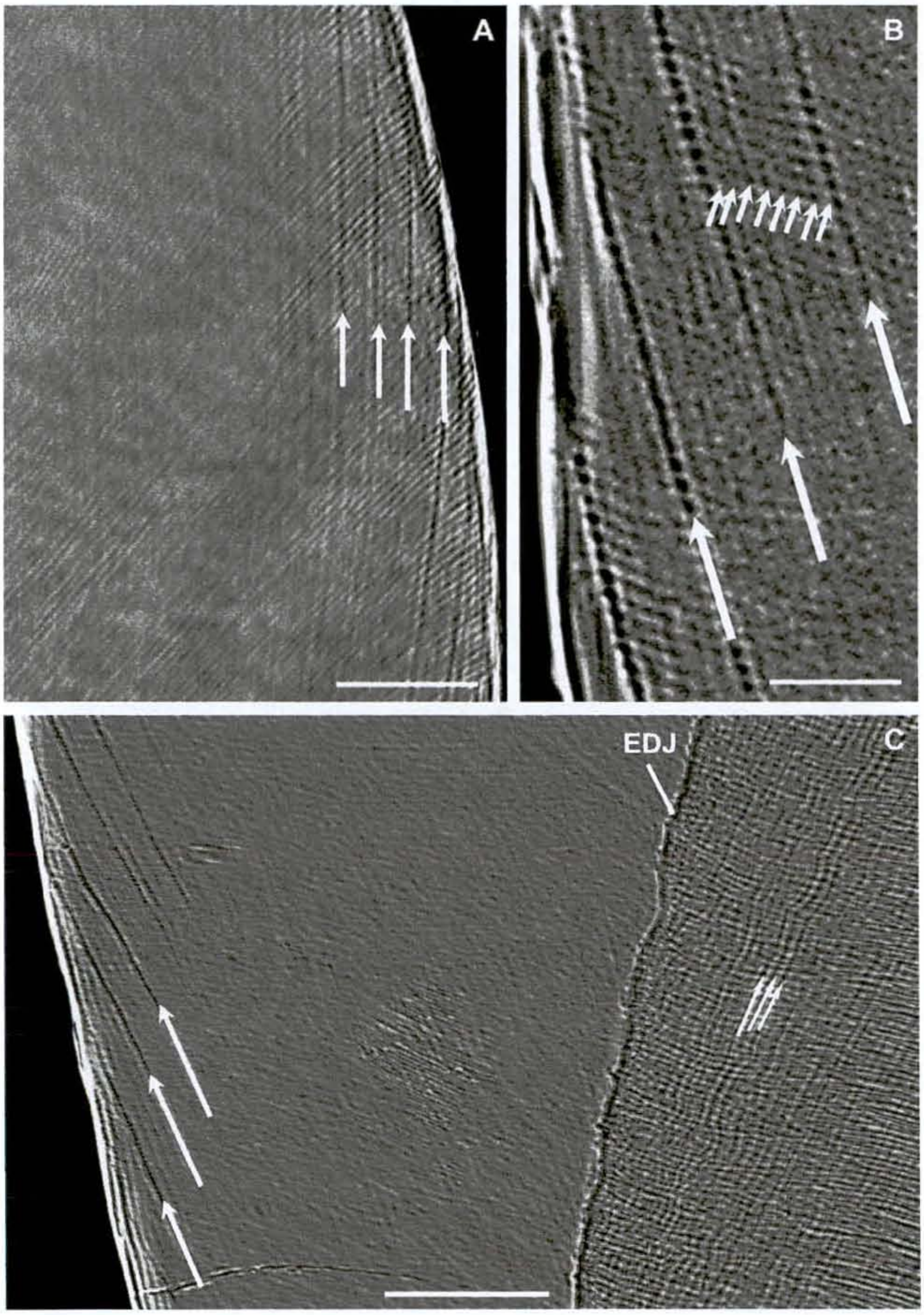

Fig. 4. Virtual imaging of microstructure in fossil hominin teeth. (A) Virtual section (70 μm thick) through *Australopithecus africanus* enamel, showing closely spaced Retzius lines (white arrows) running to the tooth surface of a partial cusp (enamel only) from Sterkfontein (EM 1011). (B) Virtual section (35 μm thick) through a molar fragment of *Paranthropus robustus* from Swartkrans (EM 2368) showing a Retzius line periodicity of eight days: eight cross-striations (small white arrows) between a pair of Retzius lines (large white arrows). (C) Virtual section (28 μm thick) of *Paranthropus robustus* enamel showing long-period lines (white arrows) in both enamel (left of enamel-dentine junction [EDJ]) and dentine (right of EDJ) of an upper molar fragment from Swartkrans (EM 1009). Scale is 100 μm in A, 50 μm in B, and 200 μm in C. The imaging protocol is the same as in Smith et al. (2007c).

2002, 2005). Using the technique presented here, which permits calculation of crown formation time, age at tooth eruption, and age at death in fossil material (e.g., Smith et al., 2007c), more precise estimates of the age at death of previously examined juvenile hominins (e.g., Bromage and Dean, 1985; Dean et al., 2001; Lacruz et al., 2005) may also facilitate greater insight into the evolution of hominin life histories.

Tafforeau (2004) demonstrated that X-ray synchrotron microtomography at medium resolution (30-μm voxel size) yields accurate measurements of virtual cross-sections when compared to physical sections of teeth (see also Olejniczak and Grine, 2006; Olejniczak et al., 2007). Here we demonstrate that submicron-resolution phase contrast SR-mCT yields faithful representations of dental microstructure, comparable to images obtained using transmitted light and confocal microscopy. Given that it is possible to virtually manipulate the slice thickness, orientation, and region of interest, this technique represents a important new step in imaging dental microstructure in fossils, particularly as it is nondestructive and can be applied to complete hominoid teeth. Current paleoanthropological applications are limited to the availability of appropriate facilities (as of now, only the European Synchrotron Radiation Facility), the amount of data generated (more than 40 gigabytes of data for a single 1.4 × 1.4 × 1.0 mm cylinder of enamel), and the need for powerful computers and image-analysis software. However, most of the modern (third-generation) synchrotrons have the ability to develop comparable imaging systems, and computer processing power is increasing rapidly.

In conclusion, recent paleoanthropological applications of X-ray synchrotron microtomography have yielded valuable information on enamel thickness and macroscopic tooth structure in fossil hominoids (Chaimanee et al., 2003; Tafforeau, 2004; Brunet et al., 2005; Chaimanee et al., 2006; Macchiarelli et al., 2006; Smith et al., 2006b; Olejniczak et al., 2008). Here we demonstrate a unique nondestructive approach to visualize internal tooth structure microscopically in two and three dimensions, which holds great promise for studies of rare fossil material. This technique reveals internal aspects of growth, including Retzius line periodicity and the presence of the neonatal line, which are key components in assessing age at death in fossil dentitions. Furthermore, it is possible to image the complex processes of dental-tissue accretion in three dimensions for extant taxa and fossils spanning several million years. Future applications of this technique will permit more precise assessments of primate evolutionary developmental biology, greater understanding of the three-dimensional structure of dental hard tissues, and may help to resolve ongoing debates over hominin life history, including reported differences or similarities between Neandertals and modern humans.

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