Cementum structure in Beluga whale teeth

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Abstract

A large fraction of the volume of Beluga whale (*Delphinapterus leucas*) teeth consists of cementum, a mineralized tissue which grows throughout the life of the animal and to which the periodontal ligaments attach. Annular growth bands or growth layer groups (GLGs) form within Beluga cementum, and this study investigates GLG structure using x-ray fluorescence mapping and x-ray diffraction mapping with microbeams of synchrotron radiation. The Ca and Zn fluorescent intensities and carbonated hydroxyapatite (cAp) diffracted intensities rise and fall together and match the light-dark bands visible in transmitted light micrographs. Within the bands of maximum Ca and Zn intensity, the ratio of Zn to Ca is slightly higher than in the minima bands. Further, the GLG cAp, Ca and Zn modulation is preserved throughout the cementum for durations > 25 yr.

Keywords: cementum; growth layer groups (GLGs); odontocete; synchrotron radiation; x-ray fluorescence; x-ray diffraction; Zn; Ca; dentin

1. Introduction

Most people know tree rings contain the growth record of these plants. Much less familiar are the analogous recording structures in the mineralized tissues of vertebrate animals [1]. Bone is one tissue which records changes in mineralization, but remodeling often eradicates early structures and preserves only the most recent changes. Tooth, provided that it is not diseased or worn away, retains the temporal sequence of tissue development in its constituent enamel, dentin and cementum. In humans and many other mammals, enamel and dentin* stop growing at a certain stage of development, and only cementum forms a record of growth through the animal's life.

Cementum covers tooth roots and anchors the periodontal ligaments attaching the tooth to the mandible or maxilla. In polished thin sections observed with transmission optical microscopy, alternating light and dark seasonal bands are often visible in cementum. The contrast between of the bands is variously attributed to differences in mineral content and/or to differences in collagen orientation. For example, Lieberman detected mineral content differences between bands in goat cementum using microradiography and, based on scanning electron microscopy (SEM) fractography, attributed changes in band contrast in transmission optical micrographs to systematic variation of collagen fiber orientation [2]. In human cementum exhibiting annular banding, however, Cool et al. observed no changes in collagen orientation in birefringence patterns and in SEM micrographs nor, using electron backscattering and spectroscopy, did they see changes in elemental composition from band to band [3]. These contradictory results suggest the need for further investigation of the microstructural,

* * Except at the border of the pulp cavity where dentin is normally added throughout life.

crystallographic and elemental bases of annular bands in mammalian teeth, and this is one motivation for the present study.

Physical anthropology makes considerable use of these cementum bands for human age determination [4], but the bands have periodicities typically in the range of 5-10 μm [5] and this hinders quantification of the microstructural bases of the contrast and, hence, interpretation of the changes in the underlying biomineralization processes. Other mammals, such as the toothed whales (odontocetes), have much more widely spaced cementum bands [6], and their study should provide insight into the more difficult to study human cementum whose band structure cannot be resolved with the $> 25 \mu\text{m}$ diameters used here. In addition, as described below, Beluga whale cementum is cellular, i.e., contains cementocytes, but still shows well defined optical banding unlike most other mammals where bands can be detected only acellular cementum.

Odontocetes range from large to small, and the several Alaskan populations of Beluga or white whales (*Delphinapterus leucas*) have been studied over a number of years, e.g. [7]. Beluga whale teeth are uniformly simple, peg-like structures with single roots and grow continuously through the life of the animal [6]. Enamel is typically worn from the tooth crowns in mature Beluga whales, and the teeth, therefore, consist of cementum with an internal zone of dentin.

Growth layer groups (GLG) form periodically in both dentin and cementum of odontocetes and, in thin sections observed optically, consist of alternating more or less opaque bands. Debate continues as to whether one or two GLGs form annually [8, 9]. Dentin GLGs are typically counted in longitudinal tooth sections using low magnification optical microscopy, and

the observed number gives a lower bound for the animal's chronological age (because some older layers, like enamel, may have worn away).

The microstructural differences underlying the optical contrast within whale GLGs received considerable attention in the 1970s [7] but relatively little since then. In the intervening years, new capabilities have become available; this paper reports application of some of these to the quantitative study of GLGs in thin sections of Beluga whale teeth. The study hypothesizes that the GLG band contrast observed in optical micrographs is mainly the product of varying mineral content and not changes in orientation of the mineral phase and underlying collagen fibrils. A second hypothesis is that Zn content tracks variations in Ca elemental and mineral phase content and that mapping of Zn fluorescent intensity provides a more sensitive indicator of changes in biomineralization than either Ca fluorescent intensity or intensity diffracted from the mineral phase.

In this study, microbeam mapping with synchrotron x-ray-excited x-ray fluorescence[†] shows maxima and minima in Ca and Zn content correspond to optical light and dark bands, respectively. Depending on the region of the tooth, different linear relationships exist between Zn and Ca concentrations. Second, synchrotron x-ray microbeam mapping shows a pattern of maxima and minima in transmission x-ray diffracted intensities (from the mineral phase carbonated hydroxyapatite, cAp) that correspond to those in the fluorescence maps, and results with different Bragg reflections are compared. The discussion concentrates first on showing the present results' consonance with the literature on Beluga whale cementum, something essential confident extension of the methods to other animals' cementum, and second on relating the

[†] Except as otherwise noted synchrotron x-ray-excited x-ray fluorescence mapping and synchrotron x-ray excited x-ray fluorescent intensities will be written omitting "synchrotron x-ray-excited".

present quantitative data to earlier conclusions on the origin of banding in cementum of other species. Suggestions for future research conclude the paper.

2. Materials and methods

Teeth of two Beluga whales (*D. leucas*) were obtained through US National Marine Fisheries Service Permit No. 932-1905/MA-009526. These animals were from the Alaskan Cook Inlet population (Table 1). A previous investigator [7] glued the teeth in sequence to a block of wood using epoxy and cut a slab 2-4 mm thick from each tooth (Fig. 1) along the central longitudinal plane.[‡] Aside from the removal procedure, air drying over a number of years and the sectioning and thinning described below, the teeth were untreated. Halves of adjacent teeth remained attached and were removed from the wood block. An Isomet-1000 saw (Buehler, Lake Bluff, IL) with a diamond wafering blade and water cooling simultaneously cut both teeth and the epoxy joining the teeth into a series of thick sections, most of which were ~150 μm thick. Longitudinal sections were cut from right teeth 2 and 3 of animal 8/14/03 (tooth 1 is adjacent to the symphyseal suture and 10 is the most posterior). Transverse sections were cut from left tooth 4 of animal 10/8/07 C1B. The free-standing as-cut sections were polished by hand mechanically using 1000 grit and then 4000 grit SiC paper. Polishing stopped when there was good visibility (via transmission optical microscopy) of features within the thin section (see Results for description of these features) and when the section began to slightly curl. The sections were rinsed briefly in ethanol, which caused the wet tooth sections to curve significantly. The wet

[‡] Prior to mounting, Vos [7] states “the jaws were thawed and boiled in water. Generally about 15 to 20 minutes of boiling time loosened the teeth enough to be pulled from the mandible. Care was taken not to boil the jaws too much so the teeth fell out, thereby losing the position of each tooth in the jaw. Teeth were immersed for about two minutes in 50% hydrogen peroxide and then scraped clean.

sections were then pressed between glass microscope slides and allowed to dry, decreasing the specimens' curvature.

Prior to x-ray mapping of the thin sections of animal 8/14/03, most of both sides of the longitudinal section of tooth 2 were covered with clear nail polish. About 4 mm (along the longitudinal axis) was left uncovered near the section's center (on matching faces of the section). After the nail polish dried, the section was placed in room temperature 0.6 N HCl and refrigerated for 30 min. Afterwards, the section was removed from the solution and thoroughly rinsed. The result was that the unprotected area was completely demineralized as determined by optical microscopy and the mapping reported in Results; only slight undercutting was observed at the edges of the covered areas.

The fluorescence data were collected at beamline 8-BM of the Advanced Photon Source (APS). Figure 2a shows a schematic of 8-BM, and data collection specifics are summarized in Table 1. Incident x-rays (10-10.5 keV) were focused to a 20 μm (horizontal) x 30 μm (vertical) wide beam using Kirkpatrick-Baez mirrors. The samples were inclined 45° to the incident beam direction, resulting in a 30 μm x 30 μm footprint on the sample surface. The specimens were raster-scanned through the beam with full x-ray fluorescence emission spectra collected at each position using a SII Vortex ME4 4-element silicon drift detector (SII NanoTechnology USA, Northridge, CA) with 0.2-1s dwell per pixel. The Vortex detector was ~3 mm from the sample.

The spectra were fitted with Gaussian models of the characteristic energy and relative intensity of the emission peaks of the elements. The data were normalized relative to the incident flux, and the MAPS software [10] converted counts to material quantity ($\mu\text{g}/\text{cm}^2$) using calibration to thin film AXO standards (Applied X-ray Optics, Dresden, Germany) using the approach outlined in [11].

Maps of diffracted intensity were recorded of the longitudinal section of tooth 2 and of tooth 3 at beamline 17-BM, APS. The scans extended beyond tooth 2 and covered part of a longitudinal section of a beaver incisor (*Castor canadensis*); the beaver enamel and dentin provided an internal reference for the Beluga cementum and dentin. Figure 2b shows a schematic of 17-BM set-up. The incident x-ray beam consisted of 17 keV photons, was defined by a 50 μm pinhole and was oriented normal to the specimen surface. An area detector (Perkin Elmer XRD 1621 area detector) positioned 401.2 mm away from specimen (and normal to the direct beam) recorded the diffraction patterns from the specimens and from a reference material (a capillary tube containing polycrystalline Si, NIST SRM-640c) mounted next to and coplanar to the tooth samples. An ionization detector mounted upstream from the specimen provided correction for changes in incident flux, and a point detector downstream from the specimen and upstream of the area detector measured intensity transmitted through specimen. Other data collection details appear in Table 1.

Corrections for area detector tilts and refinement of specimen-detector separation distance were made using diffraction patterns from the Si reference material. The 2D diffraction patterns $I(r, \eta)$ of cAp, where I is intensity, r is the radial distance from the incident beam position on the detector and η is the azimuthal angle on the detector, were then converted to 1D diffraction patterns $I(d)$ via Bragg's law. Maps of integrated intensity were produced for the 00.2, 13.0 and quadruplet[§] peaks using simple linear background subtraction and summing the total counts above background. The transmitted x-ray intensity I_{trans} was also mapped.

[§] The quadruplet reflections of 21.1, 11.2, 30.0 and 20.2 are closely spaced and are generally unresolvable in bone, dentin and cementum due the breadth of the peaks. Therefore, these reflections are treated as a single entity in this study.

The statistics of both the fluorescent intensities and diffracted intensities are governed by the standard relationship. Namely, for a signal of N counts, the uncertainty is $N^{-0.5}$ and propagates through all steps in the analyses.

The thickness of the transverse section was determined at several points using an optical contact micrometer [12, 13]. Briefly, the sample is positioned by a goniometer perpendicular to two hemispherical contact lenses made of fused silica. The lenses make mechanical contact on both sides of the sample, while the contact point of about 0.05 mm diameter can be viewed through the lenses. One lens is fixed while the opposite lens is moveable on a linear stage and possesses a plane mirror reflector. The position and thus distance between the lens tips is tracked using a two-frequency heterodyne laser interferometer, wavelength corrected in real time for air temperature, pressure, and humidity.

3. Results

Figure 3 shows optical micrographs (panels a, e, h, l) and transmitted x-ray (panels b, i, m), Ca fluorescent (panels c, f, j, n) and Zn fluorescent (panels d, g, k, o) intensity maps for the transverse and longitudinal tooth sections. Panels a-d and h-k show the entire maps; the boxes within panels a, c and d show the locations of the enlargements in e-g, respectively. All other elements (except P) had intensities an order of magnitude or more smaller than Zn and are not shown; P maps are not shown because they directly correlate with the Ca maps. Within Fig. 3, “d” labels dentin and “c” cementum; in the longitudinal section of tooth 2, “pr” and “de” identify the protected and demineralized areas. Note that Fig. 3c and f (Ca fluorescence) are shown in gray scale because the band structure was simply not visible using the color table employed for the other panels.

3.1 Transverse section

The outer surface of the tooth is on the right in Fig. 3a-g, and the cut surface near the tooth center is at the left. In the optical micrographs of the transverse section (Fig. 3a, e), alternating light and dark bands dominate the contrast; each pair is one GLG. The GLGs are slightly wavy and are centered around a point in the lower right of the micrograph. Near this center are two circular areas of light contrast described below (“pc” in Fig. 3a); these comprise the narrow pulp canal characteristic of odontocetes [14]. The GLGs are also very prominent in the Zn maps (Fig. 3d, g). Two concentric cracks are present, and the wider is labeled “c1” and the narrower “c2” in Fig. 3a. Above and to the right of the cracks, two sets of scratches are visible in Fig. 3a if one carefully inspects the image, but these scratches are not visible in the x-ray maps and do not affect the results reported below. Concentric, broad bands of contrast exist within the area defined by the cracks while the GLGs outside of the cracks are much narrower. Ignoring the modulation produced by the light/dark banding, the gray levels in Fig. 3a are smaller near the right of the image and higher in the middle and left portions; this indicates that the right side of the sample is thicker than elsewhere, something which the pattern of transmitted x-ray intensity confirms (Fig. 3b). Measured physical thicknesses of the transverse section ranged from 49 μm to 110 μm with five of the seven positions having thicknesses between 49 μm and 62 μm and the larger thicknesses tending to be on the periphery of the specimen.

Transmission optical microscopy (Fig. 4a) reveals variously oriented tubules in the volume radially enclosed by crack c2 and identified this material as dentin. The tubules appear dark against a lighter background, and most of the tubules run nearly perpendicular to the section. At the upper right of Fig. 4a (arrow), there are linear features with dark contrast and

diameters larger than the tubules; these separate the tubule-rich and the tubule-free areas (dentin and cementum, respectively) and are extrinsic fibers, i.e., buried collagen fibers that once attached the cementum to the periodontal ligaments. No tubules are found outside c2; instead cementocytes, lacunae with sparse canaliculi, are present (e.g., arrow in Fig. 4b). The material outside c2 is, therefore, cementum.

In the x-ray-based maps of the transverse sections, the non-uniform intensity complicates visibility of GLGs in all but the Zn maps. Some GLGs, however, can be observed in Ca map when it is plotted in linear gray scale (Fig. 3c). An additional complication is that the magnitudes of total Ca and transmitted intensities are much larger than the difference in signals between adjacent maxima and minima. Crack c1 is visible all three x-ray maps, but c2 is only clearly seen in the Ca map.

Starting from the light band just inside c2 in Fig. 3a, one can count 28 GLGs in the cementum. The same number can be seen in the Zn map of Fig. 3d, but contrast is too poor in the Ca map (Fig. 3c) to count all of the GLGs. There is some substructure clearly visible in the two different boxed areas of Fig. 3a,d (enlarged in Fig. 3e,g, respectively). The maxima in Zn GLGs appear to consist of double bands separated by a narrow, lower intensity zone (“%” in Fig. 3g); the same doublet GLGs are visible in the optical micrographs (“%” in Fig. 3e).

Between the crack and the pulp cavity, a pair of dentin GLGs is visible in Fig. 3a-d, albeit faintly in Fig. 3b. The dentin Ca maxima and minima intensities are comparable with those in cementum, but Zn signal in the dentin is much lower than in the cementum. The pulp cavity shows very high Zn intensity compared to the surrounding dentin GLG maxima, intensity which is comparable with the highest maxima in cementum. The Ca signal for the pulp cavity is greater

than the dentin GLG minima and comparable with the GLG maxima for both dentin and cementum.

Figure 5 shows the quantitative variation of transmitted x-ray intensity, Ca and Zn fluorescent intensities (panel a) and of optical transmitted intensity (panel b) for the positions between the red arrowheads in Fig. 3b. The position at 0 mm is at the central longitudinal saw cut through the tooth and is slightly left of the pulp cavity; the outer tooth surface is at the right and is at a position slightly greater than 11 mm. In Fig. 5, the black dashed lines linking the plots in (a) and (b) indicate the sample edges where Zn and Ca intensities drop to background and transmitted x-ray and optical intensities reach maxima. The magenta dotted lines (*I-V*) mark every fourth maximum in Zn intensity beginning from a peak a few periods away from the outer surface. The dotted lines make it clear that each Zn peak corresponds to a Ca peak, to a valley in transmitted x-ray intensity and to a maximum in optical transmitted intensity. Thus, the light bands in the optical micrographs correspond to areas of relatively high Ca content. The (Zn) peaks are more-or-less regularly spaced (with some measurable variation), and the 16 periods between dotted lines *I* and *V* average 200 μm per period.

The typical concentration of Zn within a maxima band is $8.3 \mu\text{g}\cdot\text{cm}^{-2}$, those in the two bordering minima bands are $3.6 \mu\text{g}\cdot\text{cm}^{-2}$ and $4.0 \mu\text{g}\cdot\text{cm}^{-2}$ and that measured in air is $6 \times 10^{-3} \mu\text{g}\cdot\text{cm}^{-2}$. Note that these values presume all of the mass is concentrated within a negligibly thick thin film. At the same positions, the corresponding values for Ca are 1.2×10^3 (maximum), 9.6×10^2 and 1.1×10^3 (minima) and $1.5 \mu\text{g}\cdot\text{cm}^{-2}$ (air), and for transmitted intensity 0.020, 0.026 and 0.022 and 0.071, respectively.

Figure 6a correlates Zn fluorescent intensity with Ca fluorescent intensity for each pixel within different transverse sections regions of interest (ROIs). Inset within this panel are maps

of the sections with the different ROIs are represented by different colors. Each symbol within the scatter plot represents one pixel in the map and is shown in the color of the ROI to which it belongs. The inset obscures only a few points. For a constant Ca signal, the bands with maximum Zn content had disproportionally more Zn than the minima bands (i.e., the cyan points were above the blue points in Fig. 6a).

3.2 Longitudinal section

Figure 3h-o presents data on the longitudinal sections of two teeth. The section of tooth 3 is on the left; the cementum is to the left of label “c” and dentin is to the right of “d” (Fig. 3h). The demineralized area of tooth 2 is labeled “de” and is on the bottom right (Fig. 3h); the portion protected by nail polish is labeled “pr” and is at the top right. The dotted line in Fig. 3h,i traces the border of the demineralized area. The enlargements of Fig. 3l-o are from the area bounded by the dotted box in Fig. 3j.

Aside from the GLG-related intensity variation, the transmission, Ca and Zn maps of cementum show quite uniform intensity. One can count eight GLGs in the transmission and Ca maps of tooth 3 (Fig. 3i,j). The same GLG can be seen, but less clearly in the optical micrograph and Zn maps (Fig. 3h and k, respectively). Some of the peak Zn and Ca bands of individual GLG appear to consist of closely spaced doublets; such doublets can also be seen in optical micrographs and the transmitted intensity maps. The most visible doublets in each of the enlarged area (Fig. 3l-o) are labeled with “*”.

The un-demineralized (i.e., protected) portion of tooth 2 is cementum and contains 4 GLGs visible in the optical micrograph and the transmitted intensity and Zn maps. With this color table, the GLGs in the Ca map are not very visible. The demineralized part of tooth 2

(bottom right of Fig. 3h-k) consists of matrix (presumably type I collagen, extrinsic fibers, non-HCl-soluble non-collagenous proteins, etc.) and shows only background Ca and Zn intensities and transmitted intensity matching that of the air outside of the specimens. The portion of tooth 2 covered by nail polish shows significant Ca and Zn signal; the Zn signal from the two teeth are comparable whereas the Ca signal from tooth 2 is much lower than that from tooth 3. The Discussion details how the difference can be attributed nail polish attenuating Ca more heavily than Zn photons.

Within dentin of tooth 3, two GLGs are visible in all of the maps but the Ca (Fig. 3h-o). Further, each of these GLGs show double maxima analogous to those in cementum (some of which are labeled “\$” in various panels of Fig. 3h-o). The doublet GLGs were not observed in dentin of the transverse section although double bands visible in some places in cementum of transverse section.

Figure 6b correlates Zn fluorescent intensity vs Ca fluorescent intensity for different ROIs within the longitudinal sections. Inset within Fig. 6 are maps of the sections with the different ROIs represented by different colors; the insets obscure negligible number of points. Each symbol within the scatter plot represents one pixel in the map and is shown in the color of the ROI to which it belongs. For a constant Ca signal from tooth 2 (from the nail-polish-protected area), the bands with maximum Zn content had disproportionally more Zn than the minima bands (i.e., the green points were above the red points in Fig. 6b). In tooth 3, the Zn bands and ROI are less clearly defined than in tooth 2, and the resulting plot of Zn vs Ca intensity has greater overlap between points from maxima and minima bands (blue green and yellow, respectively) although the plot does show the same trend. Note also that the Zn signal from tooth 3 is slightly higher than that from tooth 2 while the Ca signal is about 50% larger.

Figure 7 shows diffraction patterns for the longitudinal tooth sections; the beaver dentin and enamel patterns are references for comparison with the Beluga dentin and cementum. The 2D diffraction pattern $I(r,\eta)$ from tooth 3 cementum (Fig. 7a) is typical, and only reflections from cAp are observed. For Beluga cementum, Beluga dentin and beaver dentin, the rings are continuous and complete and show little variation of intensity azimuthally. In contrast, the 2D patterns from beaver enamel (data not shown) consist of the incomplete rings typically seen with microbeams and thin section samples. Figure 7b compares 1D diffraction patterns $I(d)$ for Beluga cementum, Beluga dentin, beaver enamel and beaver dentin. Comparison of these data to earlier studies of bone and dentin [15, 16] reveals the crystalline phase is carbonated (hydroxy)apatite (cAp). As expected, only the enamel peaks are sharp enough for the reflections of the 21.1 +11.2+30.0+ 20.2 quadruplet to be resolved.** The peak positions and hence lattice parameters do not vary appreciably between the four patterns in Fig. 7b.

Figure 8a-d present diffraction-based maps from of tooth 3 where the lighter the pixel, the greater the intensity. These maps contain 66 pixels horizontally and 9 vertically. Slight drifts of intensity over time were imperfectly corrected by normalization and produced the horizontal bands of higher or lower intensity (“&” in Fig. 8a,d). The area to the left of “c” in Fig. 8a is cementum and to the right of “d” is dentin. A single pixel in the maps of diffracted intensity (labeled “#” in Fig. 8b) has anomalously high intensity and results from anomalous, high background recorded for this single frame.

In all but the 00.2 map (Fig. 8a,c,d vs b), regularly alternating maxima and minima are clear and appear at matching positions. In Fig. 8c, maxima in quadruplet integrated intensity are labeled “1”-“7” in cementum and “*i*” and “*ii*” in dentin. Figure 8e shows line profiles of the fluorescence mapping data (transmitted intensity and Ca and Zn fluorescent intensities); the

** Note the use of Miller Bravais indices for this hexagonal crystal system.

profile is measured between the yellow arrowheads in Fig. 3i. Figure 8f plots variation of intensities in diffraction-based maps (quadruplet, 13.0 and 00.2 peaks and transmitted intensity) for the positions between the two white arrowheads in Fig. 8c; these positions were chosen to match the positions used to generate Fig. 8e. The peaks in quadruplet and 01.3 intensities correspond to maxima in Ca and Zn fluorescent intensity and to minima in transmitted intensity (both diffraction and fluorescence scans), but the 00.2 intensity profile does not show such clear correspondence (see following paragraph). The period of the cementum maxima averages 200-250 μm . Thus, the measured GLG period in the longitudinal section is about the same as in the transverse section.

The seven panels of Fig. 8g are Cartesian plots of diffracted intensity (gray scale value) vs azimuthal angle η (y-axis) and d -spacing (x -axis). The plots cover the cAp 00.2 reflection and adjacent background. Sub-panels I and II of Fig. 8g show 00.2 azimuthal intensity variation for different positions in Beluga dentin; sub-panels III-VI for Beluga cementum and sub-panel VII for beaver enamel. For dentin, sub-panel I shows 00.2 for a position within a Ca/Zn/diffracted intensity GLG maximum (absorption minimum), and sub-panel II the same for a Ca/Zn diffracted intensity GLG minimum at a position 200 μm horizontally^{††} away from sub-panel I. The cementum patterns (sub-panels III-VI) are from positions spaced 150 μm apart horizontally; sub-panels III and V are from intensity maxima and IV and VI from minima.

To some extent, 00.2 diffracted intensity varies with azimuth in all of the sub-panels of Fig. 8g. This implies some degree of preferred orientation (crystallographic texture) of the cAp crystallites sampled by the beam: if the cAp crystallites were oriented randomly, diffracted intensity would be constant for all azimuthal angles. Beaver enamel (sub-panel VII) shows the greatest azimuthal variation in intensity; this is expected because enamel has highly textured cAp

^{††} Here horizontal refers to the direction across the maps in Fig. 8.

[17]. The dentin patterns also show some cAp preferred orientation both from the GLG band maximum (sub-panel I) and minimum (sub-panel II). Sub-panels III-VI show less texture than in dentin. The 00.2 reflections from GLG maxima (sub-panels III and V) both show weaker texture than in dentin. One minimum position had texture approaching that in dentin (sub-panel IV) while the other (sub-panel VI) showed nearly isotropic intensity.

4. Discussion

The uncertainty in the Ca and Zn fluorescent intensities and cAp diffracted intensities is dominated by counting statistics. This uncertainty is quite a bit smaller than the variation in intensities between adjacent maxima and minima. The mapping of the bands making up the GLGs is further aided by the fact that they are continuous (2D in thin sections and 3D in the volume) in the main with only a small amount of substructure. Therefore, the identification of the Beluga GLGs is quite robust by the synchrotron x-ray methods.

4.1 Modulation of Ca and Zn content

The Ca and Zn fluorescent intensities and cAp diffracted intensities rise and fall together. Further, the GLG Ca and Zn modulation is preserved throughout the cementum, i.e., for durations > 25 yr, and this is strong evidence that a great deal of the Zn is immobilized during growth. Given that the vast majority of Ca in cementum and dentin is found within the mineral phase, this suggests that most of the Zn is also within the cAp lattice or decorates the surface of the mineral crystallites. The cAp nanoplatelets' small dimensions and very large total surface areas - $\sim 3 \times 10^5 \text{ mm}^2$ cAp surface area per mm^3 of bone [18] and not much different for cementum – can accommodate a large number of Zn atoms or Zn-containing macromolecules.

Mild HCl treatment removes Ca and Zn to background levels which is consistent with these elements being associated with cAp (alternatively some of Ca or Zn may be part of other HCl soluble entities such as proteins).

If Zn is incorporated to different extents within cAp in the maxima and minima GLG bands (Fig. 6), changes in the cAp lattice parameters may be large enough to measure [19]. Rietveld analysis of entire diffraction patterns can provide precise quantification of small changes in lattice parameter and small changes in crystallographic texture [20]. Applied to the array of patterns acquired in this study, Rietveld refinement possibly could reveal subtle variation in lattice parameters across GLGs in the Beluga whale cementum. If measurable differences exist, that would be consistent with the variation of Zn content; if differences could not be detected, this would establish an upper limit for the Zn variation that could be present within the mineral. This would also help determine the extent to which Zn is associated with the mineralization front (i.e., is transient) or is immobilized within the cAp crystal lattice.

The 00.2 map of diffracted intensity in tooth 3 (Fig. 8b) does not show the same clear set of GLG bands that are seen in the other two diffraction maps and the map of transmitted intensity (Fig. 8a,c,d). The cementum of this specimen possesses relatively weak 00.2 crystallographic texture, and this texture varies with position. It can be stronger or weaker for positions in bands of diffracted intensity maxima (sub-panels 3 vs 5 of Fig. 8g) and for positions in minima bands (sub-panels 4 vs 6 of Fig. 8g). The spatial pattern of 00.2 texture does not correspond, therefore, to the spatial distribution of crystalline material (13.0 and quadruplet maps, Fig. 8c,d), and this appears to be the origin of the poorly visible GLGs in the 00.2 diffracted intensity map (Fig. 8b). As the 00.2 reflection is the strongest indicator of cAp crystallographic texture, these results imply that texture is not a main factor in GLG visibility in

optical micrographs, contrary to discussions on odontocetes [21] and conclusions based on electron-excited energy dispersive spectroscopy of bear cementum [22].

In the maps of Fig. 3, the maxima and minima bands of the cementum GLGs comprise roughly equal fractions of each the GLG period. This cannot be taken to mean that cementum is added at a constant rate throughout the year. Further, Fig. 6 shows the ratio of Zn to Ca signal is somewhat greater in the maxima than in the minima bands of the GLGs; this suggests that the mineralization process varies slightly during the year. Mapping with micrometer or smaller x-ray beams of somewhat thinner specimens would be required to illuminate details of the annual mineralization cycle. Along with smaller beams, one could use the measured variations across the outermost, partial GLG (outermost partial peak at ~9.9 mm in Fig. 5a and ~1.5 mm in Fig. 8e) and known date of death to calibrate composition and mineral level with seasonal activity.

4.2 Relative amounts of Zn and Ca and origin(s) of Zn

Several observations in the present study and from the literature report the relative amounts of Ca and Zn within mammalian mineralized tissue. The maximum Zn fluorescent signal in the present study is about 0.4% of that of Ca; the relative amount of Zn is actually less than this because Ca K-radiation is much more heavily attenuated by Ca-based mineralized tissues than is Zn. The areas around the small pulp cavity in the transverse section produced the highest Zn signal in this map, despite being in the thinnest portion of specimen. In the longitudinal section of tooth 3, there is two to three times more Zn in cementum than in dentin. In the Beluga transverse section near the cracks (where thickness does not vary much), the GLG bands of maximum intensity contain two to three times more Zn in cementum than in those in dentin. Stock et al. [23] used a 250 nm diameter beam and found Zn signals from bovine and

equine peritubular dentin (PTD) peaked at 2.4 to 3.2 times that in the intertubular dentin (ITD); this suggests that PTD and cementum contain comparable concentrations of Zn. In human mineralized cartilage and the adjacent bone, synchrotron x-ray microbeam mapping found Zn signals 3.7% and 1.2% of Ca, respectively; significant Zn and Ca gradients were also observed [24]. Together, these data suggest Zn is elevated where mineralization is active and some Zn is transient and some fixed in the tissue.

If Zn is elevated at bone, dentin and cementum mineralizing interfaces, then one would have expected to see increased Zn signal at the outer cementum surfaces in the Beluga teeth. A peak in Zn signal was not observed at the tooth surfaces in the present maps, but this may result simply from a high Zn zone being very narrow compared to the beam diameter (30 μm). The resulting averaging with normal composition cementum most likely masks the Zn peak. The authors predict mapping the same teeth with a sub-micrometer diameter x-ray beam will reveal Zn strongly concentrated at the Beluga cementum surface. Further, the authors expect such concentrations at the cementum surfaces of all vertebrates with apatitic teeth.

Zinc has long been known as a very important trace element in apatitic hard tissues (cementum, dentin, bone) [25], but zinc's role in mineralization of teeth has not appear to have been studied extensively. Zinc is found in many factors important in biomineralization, and, in teeth, the following have been documented: in dentin, the alkaline phosphatase superfamily [26], osterix [27, 28] and matrix metalloproteinases MMP-2, -8, -9 and -20 [29-31]; in cementum, MMP-2 and -8 [32]; in tooth pulp, MMP-13 [33]. Immunohistochemistry would be required to determine which of these (if any) are responsible for the Zn observed in the Beluga cementum.

The x-ray fluorescence maps provide a very sensitive map of total Zn, regardless of its association. Rietveld refinement of lattice constants from the cAp diffraction patterns and use of

a calibration curve - lattice constant vs hAp Zn content [19] - should reveal how much Zn is incorporated in the crystal phase of cementum. Because typical immunohistochemistry studies are only semi-quantitative, knowledge of total Zn and of Zn in the mineral phase is important for establishing upper bounds for the organic-related Zn. Note that even though Zn fluorescent intensity rises and falls in concert with cAp diffracted intensity, this does not rule out an association of Zn with the organic constituents of cementum: the concentration of Zn-containing macromolecules may scale with cAp content (e.g. because they are controlling the mineralization) and these macromolecules may have been trapped by subsequent mineralization.

4.3 Growth layer groups and age of animal 10/8/07

The GLG period in both the longitudinal and transverse sections were comparable: 200-250 μm in the former and 200 μm in the latter. Given that the beam sizes were 30 μm for fluorescent mapping and 50 μm for diffraction mapping and that there is no guarantee that the section plane was normal to the GLGs, it is difficult to conclude that the two measured cementum periods differ. In the transverse section, GLGs were broader in dentin than in cementum, but in the longitudinal section the dentin and cementum GLG thicknesses were comparable. Presumably the difference is the result of the transverse sectioning plane intersecting the dentin bands much more obliquely than in the longitudinal section.

The transverse section reveals 28 GLGs, and age at death must have been greater than 28 years. This number is within the range of dentin GLGs of *D. leucas* counted in recent studies [8, 9] and consistent with reported life span in the wild of 35-50 years [34]. Therefore, this study's results are reasonable.

In Beluga teeth, a single GLG corresponds to one years' growth, but sometimes two GLGs have been reported per year, even in studies where great effort went into minimizing inter-observer and sample preparation variability. The present data suggest that GLG waviness and substructure in both cementum and dentin may be the origin of reports of more than one GLG per year. In the transverse section (Fig. 3), the bands are wavy and at some points almost merge together, indicating a potential complication in GLG counting for certain placements of longitudinal sections. Figure 3g shows clear doublet substructure in some GLG positions and less clarity in other positions, and Fig. 3m-o show a mixture of clear doublets and single, broader bands, sometimes within the same longitudinal band. The Ca and Zn profiles in Fig. 8e reveal two peaks within a single GLG, e.g., between position 1.5 and 2.0 mm.

Cementocyte lacunae (~10 μm diameter) were seen throughout the Beluga cementum and did not interfere with the imaging of the GLGs with 30-50 μm sizes x-ray beams. In other mammals, acellular and cellular cementum occupy different zones of the tooth, and the narrower GLGs can only be seen in optical microscopy within the acellular zones. The x-ray excited x-ray fluorescent mapping approach (in particular with Zn) using submicrometer beams may provide superior delineation of GLGs even when the GLGs cannot be seen optically.

4.4 Technical aspects

The portion of tooth 2 covered by nail polish shows Zn signal comparable to and Ca signal much lower than those of tooth 3. This results from the thin nail polish layer (a few tens of micrometers) on tooth 2 absorbing emitted Ca photons much more highly than Zn photons. Using the polymer polyethylene (PE) for purposes of illustration, the mass attenuation coefficient at the Ca K energy of 4 keV is 32.4 cm^2/g compared to ~2.4 cm^2/g at the Zn K α

energy of 9.7 keV [35]. As expected, the effect of the nail polish is quite small on the incident beam transmitted intensity (compare maps of tooth 2 and tooth 3 in Fig. 3i).

The variation in transmitted intensity in the transverse section maps indicates that the sample thickness varies significantly. Even where the transverse section is thickest, a significant fraction of the 10 keV incident x-radiation passes through the specimen. It is interesting to note that the highest Ca and Zn fluorescent signals emerge from the thickest parts of the specimens; this indicates some signal is reaching the fluorescence detector from all depths of the specimen. This is not surprising for Zn because the $E_{K\alpha}(\text{Zn}) = 9.7 \text{ keV}$, only slightly less energetic than in incident x-ray beam. The situation for Ca is different because $E_{K\alpha}(\text{Ca}) = 4 \text{ keV}$. Using values of mass attenuation coefficient for bone [35], 13.5% of Ca $K\alpha$ radiation escapes from a path of 81 μm through bone; 5% emerges after 122 μm .

Precise measurement of thickness are required in order to calculate local concentrations of Zn. This would be a very important extension of the present study.

4.5 Context of the present study and future prospects

Figures 5 and 8 show that peaks in Zn and Ca fluorescent intensity match peaks in cAp diffracted intensity, valleys in transmitted x-ray intensity and to maxima in optical transmitted intensity (i.e., light bands). Azimuthal variation of 00.2 diffracted intensity is present, but the crystallographic texture of cAp and of the underlying collagen does not appear to vary systematically from band to band. At least in *Beluga cementum*, the present results indicate that changes of contrast in optical are related to changes in cAp content.

It might be argued that the variations in fluorescent and diffracted intensities, in transmitted x-ray intensity and in optical transmitted intensity might be produced by local

variations in section thickness, produced perhaps by differential polishing related to varying microstructure across each GLGs. The physical thickness measurements described above could not address this possibility: the probe averages over too much area, and the positioning is too imprecise. Transmitted light observations show that the GLG bands appear at the same positions in the as-cut sections and in the sections thinned by polishing. Differential material removal during polishing with SiC paper is not inconceivable, but it is difficult to imagine sawing producing such an effect. Further, the dry polishing used in the present study is unlikely to produce banding, given that protocols for etching to reveal GLG layers via surface relief in SEM require 3 hr exposure to 5% formic acid [36]. This means that x-ray signals corresponding to the light and dark optical bands are arising from the volume of the material not variations in thickness.

The thickness of the transverse section varies slowly, but this does not affect interpretation of the trends in Ca and Zn content. Thickness changes little across a Ca (Zn) fluorescent intensity maximum, i.e., between adjacent minima, and all of the maxima have fairly similar heights relative to the neighboring minima. Further, the longitudinal section (which has roughly constant thickness, except for the demineralized area) shows the same pattern of Ca and Zn maxima and minima.

The fluctuations in Zn fluorescent intensity are a higher percentage of the total signal than are the variations in diffracted intensities, in Ca fluorescent intensity and in transmitted x-ray intensity. This makes Zn a good marker for studying variation in mineral content of cementum and other mineralized tissues. Note that multiple studies have found increased Zn concentration at or near mineralization fronts in a variety of tissues [23, 24] including in

cementum affected by pathological processes [37]. Study of Zn and its origins may, therefore, also add quantitative insight into fundamental mineralization processes.

The present study shows that diffraction and fluorescent mapping with small diameter synchrotron x-ray beams provides valuable information on cementum in Beluga whales. The new approach offers a valuable alternative to optical microscopy or SEM for studying GLGs. The beam diameters used here (30 μm for fluorescence and 50 μm for diffraction mapping) are appropriate for the GLG period and sample thickness of the whale tooth sections but are much too large to provide meaningful information for other mammals. Cementum GLGs for bovine, reindeer, human or many other mammals have periods on the order of 5-10 μm , e.g. [1,5], and much smaller diameter beams would be required. Stock et al. [23], for example, used a ~250 nm wide beam and similar x-ray energies to map differences in Ca and Zn composition and in cAp content and crystallographic texture around 1-2 μm diameter tubules, i.e., for PTD vs ITD. Fluorescence and diffraction mapping with a 250 nm beam should, in suitable specimens, delineate the fine details of GLGs in human and ruminant cementum and improve age and season of death determinations. The authors expect, therefore, that mapping with sub-micrometer beams will change understanding of cementum mineralization.

Conflicts of Interest

The authors have no conflicts of interest to report.

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References

1. Klevezal GA. Recording Structures of Mammals. Rotterdam: Balkema; 1996.
2. Lieberman DE. Life history variables preserved in dental cementum microstructure. *Science* 1993; 261: 1162-1164.
3. Cool SM, Forwood MR, Campbell P, Bennett MB. Comparisons between bone and cementum compositions and the possible basis for their layered appearances. *Bone* 2002; 30: 386-392.
4. Grosskopf B, McGlynn G. Age diagnosis based on incremental lines in dental cementum: A critical reflection. *Anthrop Anz* 2011; 68: 275-289.
5. Grosskopf B. Individualaltersbestimmung mit Hilfe von Zuwachsringen im Zement bodengelagerter menschlicher Zähne. *Z Rechtsmed* 1990; 103: 351-359.
6. Perrin WF, Myrick AC Jr., editors. Age Determination of Toothed Whales and Sirenians. Cambridge: International Whaling Commission; 1980: Special Issue 3.
7. Vos DJ. Cook Inlet Beluga Age and Growth. Anchorage: Alaska Pacific University MS Thesis; 2003.
8. Hohn AA, Lockyer C. Growth layer patterns in teeth from two known-history Beluga whales: Reconsideration of deposition rates. *Int Whaling Comm SC51*, 1999. SM4.
9. Lockyer C, Hohn AA, Doidge DW, Heide-Jorgensen MP, Suydam R. Age determination in Belugas (*Delphinapterus leucas*): A quest for validation of dentinal layering. *Aquatic Mammals* 2007; 33: 293-304.
10. Vogt S. MAPS: A set of software tools for analysis and visualization of 3D X-ray fluorescence data sets. *J Phys IV* 2003; 104: 635-638.

11. Twining BS, Baines SB, Fisher NS, Maser J, Vogt S, Jacobsen C, Tovar-Sanchez A, Sanudo-Wilhelmy SA. Quantifying trace elements in individual aquatic protest cells with a synchrotron x-ray fluorescence microprobe. *Anal Chem* 2003; 75: 3806-3816.
12. Jacobsen, SD. Optical Contact Micrometer. US Patent No. 8810904, 2014.
13. Chang Y-Y, Jacobsen SD, Kimura M, Irifune T, Ohno I. Elastic properties of transparent nano-polycrystalline diamond measured by GHz-ultrasonic interferometry and resonant sphere methods. *Phys Earth Planetary Interiors* 2014; 228: 47–55.
14. Scheffler VB, Myrick AC, Jr. A review of studies to 1970 of growth layers in the teeth of marine mammals. In: Perrin WF, Myrick AC Jr., editors. *Age Determination of Toothed Whales and Sirenians*. Cambridge: International Whaling Commission; 1980, Special Issue 3. p. 51-63.
15. Almer JD, Stock SR. Internal strains and stresses measured in cortical bone via high-energy x-ray diffraction. *J Struct Biol* 2005; 152: 14-27.
16. Almer JD, Stock SR. Loading-related strain gradients spanning the mature bovine dentinoenamel junction (DEJ): Quantification using high energy x-ray scattering. *J Biomech* 2010; 43: 2294-2300.
17. Al-Jawad M, Streuwer A, Kilcoyne SH, Shore RC, Cywinski R, Wood DJ. 2D mapping of texture and lattice parameters of dental enamel. *Biomater* 2007; 28: 2908-2914.
18. Stock SR. The mineral-collagen interface in bone. *Calcif Tiss Int* 2015; 97: 262-280.
19. Matsunaga K, Murata H, Mizoguchi T, Nakahira A. Mechanism of incorporation of zinc into hydroxyapatite. *Acta Biomater* 2010; 6: 2289-2293.
20. McCusker LB, Von Dreele RB, Cox DE, Louër D, Scardi P. Rietveld refinement guidelines. *J Appl Cryst* 1999; 32: 36-50.

21. Myrick AJ Jr. Examination of layered tissues of odontocetes for age determination using polarized light microscopy. In: Perrin WF, Myrick AC Jr., editors. Age Determination of Toothed Whales and Sirenians. Cambridge: International Whaling Commission; 1980: Special Issue 3, p105-112.
22. Smith KG, Strother KA, Rose JC, Savelle JM. Chemical ultrastructure of cementum growth-layers of teeth of black bears. *J Mammal* 1994; 75:406-409.
23. Stock SR, Deymier-Black AC, Veis A, Telser A, Lux E, Cai Z. Bovine and equine peritubular and intertubular dentin. *Acta Biomater* 2014; 10: 3969-3977.
24. Zoeger N, Streli C, Wobrauschek P, Jokubonis C, Pepponi G, Roschger P, Hofstaetter J, Berzlanovich A, Wegrzynek D, Chinea-Cano E, Markowicz A, Simon R, Falkenberg G. Determination of the elemental distribution in human joint bones by SR micro XRF. *X-Ray Spectrom* 2008; 37: 3-11.
25. Vincent J. Microscopic aspects of mineral metabolism in bone tissue with special reference to calcium, lead and zinc. *Clin Orthop Rel Res* 1963; 26: 161-175.
26. Bobyr E, Lassila JK, Wiersma-Koch HI, Fenn TD, Lee JJ, Nikolic-Hughes I, Hodgson KO, Rees, DC, Hedman B, Herschlag D. High-resolution analysis of Zn^{2+} coordination in the alkaline phosphatase superfamily by EXAFS and x-ray crystallography. *J Mol Biol* 2012; 415: 102-117.
27. Chen S, Gluhak-Heinrich J, Wang YH, Wu YM, Chuang HH, Chen L, Yuan GH, Dong J, Gay I, MacDougall M. Runx2, Osx, and DSPP in tooth development. *J Dent Res* 2009; 88: 904-909.
28. Hirata A, Sugahara T and Nakamura H. Localization of Runx2, Osterix and osteopontin in tooth root formation in rat molars. *J Histochem Cytochem* 2009; 57: 397-403.

29. Yamakoshi Y, Hu JC, Iwata T, Kobayashi K, Fukae M and Simmer JP. Dentin sialophosphoprotein is processed by MMP-2 and MMP-20 *in vitro* and *in vivo*. J Biol Chem 2006; 281: 38235-38243.
30. Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T and Tjaderhane L. Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. Arch Oral Biol 2007; 52:121-127.
31. Mazzoni A, Pashley DH, Tay FR, Gobbi P, Orsini G, Ruggeri A Jr, Carrilho M, Tjaderhane L, Di Lenarda R and Breschi L. Immunohistochemical identification of MMP-2 and MMP-9 in human dentin: Correlative FEI-SEM/TEM analysis. J Biomed Mater Res 2009; 88A: 697-703.
32. Maruya Y, Sasano Y, Takashashi I, Kagayama M, Mayanagi H. Expression of extracellular matrix molecules, MMPs and TIMPs in alveolar bone, cementum and periodontal ligaments during rat tooth eruption. J Electron Microsc 2003; 52: 593-604.
33. Sulkala M, Pääkkönen V, Larmas M, Salo T and Tjäderhane L. Matrix metalloproteinase-13 (MMP-13, Collagenase-3) is highly expressed in human tooth pulp. Conn Tiss Res 2004; 45: 231-237.
34. NOAA Fisheries. Beluga Whale (*Delphinapterus leucas*).
<http://www.fisheries.noaa.gov/pr/species/mammals/whales/beluga-whale.html>. Accessed July 17, 2015.
35. Hubble JH, SM Seltzer. Tables of X-ray mass attenuation coefficients and mass energy-absorption coefficients from 1 keV to 20 MeV for elements $Z = 1$ to 92 and 48 additional substances of dosimetric interest. NISTIR 5632.
<http://www.nist.gov/pml/data/xraycoef/index.cfm> Accessed July 17, 2015.

36. Hohn AA. Analysis of growth layers in the teeth of *Tursiops truncatus* using light microscopy, microradiography and SEM. In: Perrin WF, Myrick AC Jr., editors. Age Determination of Toothed Whales and Sirenians. Cambridge: International Whaling Commission; 1980: Special Issue 3, p155-163.
37. Djomehri SI, Candell S, Case T, Browning A, Marshall GW, Yun W, Lau SH, Ho SP. Mineral density volume gradients in normal and diseased human tissues. PLoS One 2015; 10:e0121611.

Table 1. Specimen description and data collection parameters. Adult whale 8/14/03 was 3.38 m long, and information for 10/8/07 is not available.

Sample	L/T*	Beam line	F/D [#]	E keV	Dia. μm	Step size (μm)		number		Time sec
						x	y	x	y	
<u>8/14/03</u>										
tooth 2	L	8-BM	F	10	30	30	30	111	251	1
		17-BM	D	17	50	50	200	100	9	57
tooth 3	L	8-BM	F	10	30	30	30	111	251	1
		17-BM	D	17	50	100	100	50	2	57
<u>10/8/07 C1B</u>										
tooth 5	T	8-BM	F	10	30	25	25	101	408	1

* Section orientation: L – longitudinal, T – transverse.

[#] Data collection: F – fluorescence, D – diffraction in transmission geometry.

Table 2. (top) max/min “intensities” in the plots of Fig. 3. “opt.” is optical micrograph, “trans.” is x-ray intensity transmitted and is normalized by the intensity measured by ionization detector before the specimen and the units of Ca and Zn fluorescent intensity are $\mu\text{g cm}^{-2}$.

Panel	Transverse section				Longitudinal section			
	opt. a, e	trans. b	Ca c, f	Zn d, g	opt. h, l	trans. i, m	Ca j, n	Zn k, o
Max	-	0.087	1486	8.91	-	0.152	2951	18.7
Min	-	0	-80	1.30	-	0.033	908	1.6

Figure captions

Figure 1. (a) Teeth from one side of a mandible cemented to a wood block. Each tooth is positioned with the curved side down. A central slab was cut from the center of each tooth (Vos 2003), and the 2-4 mm width kerf was not available for this study. Two tooth halves at “#” removed for sectioning. Tooth at “*” enlarged in (b) to show the longitudinal slice plane and in (c) to show the transverse slice plane. (d) Schematic of longitudinal tooth cross-section with cementum shown as light gray and dentin as dark gray. The pulp cavity is labeled “\$”.

Figure 2. Experimental set-ups for x-ray data collection. The sample is rastered across the beam in directions x and y . (a) X-ray fluorescence microprobe at station 8-BM-B, APS. The sample is in a helium (He) filled chamber and the fluorescent intensities are measured by detector D_1 which is in contact with a kapton window in the chamber. (b) X-ray micro-diffraction set-up at station 17-BM, APS. The area detector D_1 measures scattered intensity at angles 2θ . Both panels show the incident intensity and transmitted intensity detectors (D_0 and D_2 , respectively).

Figure 3. Comparison of optical micrographs, transmitted intensity and fluorescent intensity maps. The color table used for all of the x-ray images, except the Ca maps of the transverse section (c, f), is in an open space near the center of the figure. Entire transverse section (overview, scale bar in (b) is 1 mm): (a) Optical micrograph; (b) Transmitted x-ray intensity map; (c) Ca fluorescent intensity map; (d) Zn fluorescent intensity map. Transverse section (enlarged area shown in (a, c and d), 1 mm scale bar above the color bar between (e) and (f)): (e) Optical micrograph; (f) Ca fluorescent intensity map; (g) Zn fluorescent intensity map. Longitudinal sections (overview, scale bar in (j) is 1 mm): intact section of tooth 3 (left); tooth 2 (right) mineralized area protected by nail polish (“pr”, top), demineralized area (“de”, bottom).

(h) Optical micrograph. The red fiducial blob is from a marker pen and was used as a guide when the nail polish was applied. (i) Transmitted x-ray intensity map; (j) Ca fluorescent intensity map; (k) Zn fluorescent intensity map. Enlarged areas of longitudinal section of tooth 3 (the box in (j) defines the area, scale bar in (n) is 1 mm): (l) Optical micrograph; (m) Transmitted x-ray intensity map; (n) Ca fluorescent intensity map; (o) Zn fluorescent intensity map. Labels are: d- dentin, c – cementum, cr – crack. Arrowheads in (b) and in (i) mark the positions of 1D profiles shown in Fig. 5.

Figure 4. Optical micrographs. (a) dentin tubules and (b) cementocyte lacunae. The position of (a) is underneath “d” in Fig. 3a; that of (b) is less than 1 mm to the right of “c” in the figure. The scale bars are 100 μm and 20 μm , respectively.

Figure 5. One-dimensional profiles of optical density, transmitted x-ray intensity, Ca and Zn fluorescent intensities in the transverse section. (a) 1D profiles between the arrowheads on the transverse section (Fig. 3b) showing Ca and Zn fluorescent and transmitted intensities. (b) Variation of optical density at positions matching those in (a). The vertical dashed and dotted lines are added to guide the eye when comparing the peak positions. The other symbols are defined in the text.

Figure 6. Scatter plot Zn vs Ca for the maps shown in Fig. 3. (a) Scatter plot of Zn vs Ca intensities for the transverse section. Each symbol represents one pixel in the map. Points from within different ROIs shown in the inset are indicated by the various colors. (b) Scatter plot of Zn vs Ca intensities for the longitudinal sections. Points from within different ROIs are shown in the inset. In both (a) and (b), the insets obscure a negligible number of points. indicated on the Zn map of the transverse tooth section.

Figure 7. Diffraction patterns. (a) Typical 2D pattern of cementum from tooth 3. The darker the pixel, the greater the intensity. The 00.2 and quadruplet q (21.1+11.2+30.0+20.2) diffraction rings are labeled. Azimuthal angles $\eta = 0^\circ$ and 90° are indicated as is the incident beam position D2. (b) Comparison of 1D diffraction patterns from Beluga cementum and dentin with beaver dentin and enamel. The intensities are in units of 10^3 counts, the enamel intensities have been divided by three to keep all of the patterns on the same scale and the patterns have been shifted vertically for clarity. The 00.2, q and 13.0 diffraction peaks (labeled) are used in the maps of Fig. 8.

Figure 8. Maps and line profiles for diffraction data on longitudinal sections of tooth 3. The maps are 8-bit, linear gray scale images, and the maximum and minimum values in the maps are given by [min, max] for each panel. Maps with: (a) Transmitted intensity, [50, 255]. (b) 00.2 integrated intensity, [35, 130]. The scale bar is 1 mm long. (c) 21.1+11.2+30.0+20.2 integrated intensity, [50, 200]. The arrowheads mark the position of the 1D profiles in (e, f). (d) 13.0 integrated intensity, [50, 220]. (e, f) 1D profiles from the fluorescent and diffraction maps, respectively. The horizontal axis is position in millimeters, and the vertical is intensity. The vertical dashed lines are added to guide the eye when comparing the peak positions. The other symbols are described in the text. (g) Cartesian plots of diffracted intensity (gray scale value) vs azimuthal angle η (y-axis) and d -spacing (x-axis) covering the cAp 00.2 reflection and adjacent background. Sub-panels I and II are of Beluga dentin; sub-panels III-VI of Beluga cementum and sub-panel VII of beaver enamel. Further details appear in the text.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8