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Growth of second stage mineral in *Lytechinus variegatus*

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Abstract

Purpose and Aims: Sea urchin teeth consist of calcite and form in two stages with different magnesium contents. The first stage structures of independently formed plates and needle-prisms define the shape of the tooth, and the columns of the second stage mineral cements the first stage structures together and control the fracture behavior of the mature tooth. This study investigates the nucleation and growth of the second stage mineral.

Materials and Methods: Scanning electron microscopy and synchrotron microComputed Tomography characterized the structures of the second phase material found in developing of *Lytechinus variegatus* teeth.

Results: Although column development is a continuous process, defining four phases of column formation captures the changes that occur in teeth of *L. variegatus*. The earliest phase consists of small 1–2 μm diameter hemispheres, and the second of 5–10 μm diameter, mound-like structures with a nodular surface, develops from the hemispheres. The mounds eventually bridge the syncytium between adjacent plates and form hyperboloid structures (phase three) that appear like mesas when plates separate during fracture. The mesa diameter increases with time until the column diameter is significantly larger than its height, defining the fourth phase of column development. Energy dispersive x-ray spectroscopy confirms that the columns contain more magnesium than the underlying plates; the ratios of magnesium to calcium are consistent with compositions derived from x-ray diffraction.

Conclusion: Columns grow from both bounding plates. The presence of first phase columns interspersed among third stage mesas indicates very localized control of mineralization.

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Conflict of Interest Statement

The authors have no conflicts of interest.

Keywords

teeth; sea urchin; biomineralization; calcite; mechanical properties

Introduction

Sea urchins have evolved highly functional teeth comprised of a wretched structural material, calcite, a crystalline form of calcium carbonate. The focus here is not the chemistry and microstructure underlying the remarkable mechanical properties of urchin teeth but rather the details of nucleation and growth of one of the structures critical to achieving these properties. Mineralization and properties of urchin teeth are of intrinsic interest, but, because the macromolecules implicated in controlling urchin tooth mineralization are homologous with those active in vertebrate teeth (1), this calcium carbonate biomineralization system also provides an important window into mineralization of the calcium phosphate vertebrate teeth.

Sea urchins have complex calcite skeletons, and their teeth differ in structure from the stereom comprising the rest of the skeletal elements or ossicles. Stereom is a highly fenestrated single crystal of calcite, and the volume percent of stereom within sea urchin ossicles can vary from 30 to 90% depending on location (2). Teeth are also single crystals of calcite¹, grow continuously and mineralize in two stages. The first-forming mineral consists of plates (primary, secondary and, in some taxa, carinar process), the lamellae-needle complex and needle-prisms that form in spaces between cellular syncytia, and the second fills the space between adjacent first stage mineral elements (3, 6–13). The first stage mineral elements develop initially in the plumula (most aboral portion of the tooth); and the second stage mineral (in the form of columns or disks) subsequently links the plates and prisms together.

All of the calcite in the sea urchin tooth contains significant Mg substituted for Ca, i.e., $\text{Ca}_{1-x}\text{Mg}_x\text{CO}_3$ with $0.1 < x < 0.15$ in the first stage mineral and with $0.3 < x < 0.4$ in the second stage (4, 5, 14). Several studies show the columns are crystallographically co-oriented with the underlying first stage mineral (4, 5, 15). The literature terms the second stage mineral as “polycrystalline disks”, e.g. (3, 8–12), but Stock (16) suggests that the columns have an epitaxial relationship with the underlying “substrate”, much like AlGaAs/GaAs superlattices grown by molecular beam epitaxy, and that the tooth is a compositionally modulated crystal.

The second stage mineral differs from the first stage mineral not only in when it forms and in its composition (very high Mg content ~ 0.3–0.4 mole fraction vs the high Mg content ~ 0.1–0.2 mole fraction for the first stage mineral (15)) but also in its mechanical/fracture properties. Hardness and toughness differ for the two materials (17) and differ with position within the flange and keel (10, 12); and fracture tends to follow the interface between first and second stage mineral or to cross plates leaving a glass-like fracture surface (12, 18).

¹More precisely, each tooth is a bi-crystal with a small disorientation of 5–10° across the tooth’s plane of symmetry, e.g. (3–5).

This toughens the material and, because of the orientation of the primary and secondary plates, produces a self-sharpening incisal end. Stock (16) has studied the later stages of column growth, when columns fill most of the channel between plates, and has provided an estimate of the rate of very high Mg calcite addition at that stage of tooth maturation. The early stages of column growth, however, have not been studied in equal depth, and this paper examines column morphology up to the point where the columns begin to merge.

Figure 1 schematically illustrates the growth of first and second stage minerals. Panel (a) shows a series of plates (first stage mineral) growing in syncytial spaces, and (b) pictures the situation after the plates have completely filled the space between syncytia. Panels (c-h) show a single multi-nucleated cell syncytium and its bounding plates; different stages of growth of the second stage mineral appear. Note that (c) and (e) show growth from only one side, and (d) and (f) present the alternative where growth across the syncytium occurs from both bordering plates. One aim of the present study is to determine whether columns grow as described in (c,e) or in (d,f). A second aim is to examine how the morphology of the second stage mineral columns change as the columns mature.

In the present study of teeth of *Lytechinus variegatus* (Lamarck, 1816), a species of the family Toxopneustidae (sea urchins with keeled teeth and known as camarodonts), scanning electron microscopy (SEM) reveals several distinct column microstructures in fractured plates, and all of these developmental phases can be seen on the same plate within the span of a few tens of micrometers. Further, the columns nucleate and grow on both sides of the channel between adjacent plates. Energy dispersive spectroscopy reveals that all of column developmental stages have the same elemental signature: all stages have similar Mg content, content higher than that of the plates on which they perch, and the column compositions match results of x-ray diffraction reported previously. Synchrotron microComputed Tomography (microCT) with 280 nm isotropic volume elements (voxels) demonstrates that the structures seen on fractured plates (with SEM) are present within the undamaged *L. variegatus* tooth.

Nucleation of the mineral, whether from solution or from solid state transformation of an amorphous precursor phase or via another mechanism, although a very important topic, is outside the scope of this paper. The details of growth of the second stage mineral (columns), the subject of this paper, are also not minutiae but rather are essential to understanding the fundamental processes completing urchin tooth mineralization and producing functional teeth. Sea urchin teeth grow continuously to replace material worn from the adoral surfaces, and existing plates and prisms are pushed adorally as new plates are added in the aboral plumula. Urchin tooth mineralization thus is a continuum consisting of many processes operating sequentially along the tooth's aboral-adoral axis (e.g. nucleation and growth of primary plates in separate syncytial spaces, each with identical crystallographic orientation; growth of secondary plates at the lateral ends of primary plates; nucleation of prisms with the tooth; nucleation and growth of second stage mineral). Understanding what initiates a certain process or switches off another is a fundamental part of explaining how biomineralization proceeds. Detailed description of developmental stages of column growth in *L. variegatus* allows one to infer that this stage of mineralization is under localized control, something quite different from the controls operating during growth of first stage

mineral (primary and secondary plates). Further, accurate and predictive models of column growth must be based on data on the stages through which they pass.

Materials and Methods

Adult *L. variegatus* sea urchins were obtained from Panacea, FL, USA (Gulf Specimen Marine Laboratory) and placed in an aquarium. Some urchins died in the aquarium, and these urchins' teeth were scavenged from the bottom of the tank after their plumulae had disintegrated. Rinsed and dried teeth were fractured, and fragments from the tooth shaft (where columns had not reached too high a density) were spread on carbon tape on SEM sample stubs. An intact tooth was used for synchrotron microCT imaging; this tooth was soaked in distilled water for 12 hours and dried in air. We note that, in the several dozen scavenged *L. variegatus* teeth we have examined by fractography or synchrotron microCT, we have not observed syncytial (soft) tissue surviving the in-tank degradation.

Very high Mg columns exposed on fractured plates were imaged with 10 or 12.5 keV secondary electrons in an environmental SEM at the Max Planck Inst. (Quanta 600 FEG). Energy dispersive spectroscopy was performed with 20 keV electrons in the environmental SEM at the Electron Probe Instrumentation Center, Northwestern Univ. (Hitachi S-4800 II); the goal was to confirm that the structures identified as early stage columns have Mg contents consistent with mature columns and prior x-ray diffraction results and not to provide precise elemental compositions.

The intact *L. variegatus* tooth was imaged using synchrotron microCT at the European Synchrotron Radiation Facility (ESRF) station ID19. Conventional synchrotron microCT, where the tooth cross-section remains entirely within the field of view of the detector, limits volume element (voxel) size to $\sim 1\ \mu\text{m}$, and much smaller voxels were needed to investigate column geometry in situ between plates. A local tomography approach, therefore, was adopted with submicrometer voxels and one wing of the flange centered on the instruments rotation stage and the rest of the tooth moving into and out of view. This approach allows higher resolution imaging of a small portion of the specimen, but the missing mass can alter values of the linear attenuation coefficient or introduce noise. Continuous rotation of the tomographic axis was used to suppress artifacts related to local tomography. The 2000 projections were recorded with 19 keV single-harmonic undulator illumination using a 16 mm propagation distance and a LSO:Tb 4.5mm scintillator (19) coupled via a 20x (0.4 NA) lens with 2.5x eye-piece to the ESRF in-house-developed FReLoN CCD detector (type 2K). Reconstruction was with isotropic $0.28\ \mu\text{m}$ voxels. Binary segmentation was used to produce a 3D view of several columns and the adjoining plates.

Results

The micrographs of Fig. 2–5 reveal four phases of second stage mineral formation, but column growth is a continuous process and such divisions are, admittedly, a bit reductionist. Figure 2 shows SEM images of the first three phases of column formation, exposed when the tooth was fractured. The first phase is a small hemispherical deposit on the underlying plate; in Fig. 2a the hemispheres (labeled “1”) are $1\text{--}2\ \mu\text{m}$ in diameter. The second phase

(labeled “2”) consists of an irregular mound with a nodular surface, and one should note that the nodule size is similar to that of the hemispheres. The mounds and hemispheres are intermingled in Fig. 2a. The mounds are not always well separated from each other (e.g., the pair of mounds above left and a second pair above right of “1” in Fig. 2a), and the hemispheres, while not clustered, are not dispersed uniformly between the mounds.

The third phase spans column development from the point where the columns just bridge the syncytium to where the columns grow more much wider than they are tall (phase 4). In fractured specimens such as those that appear in Fig. 2–6, the columns have flat tops where they separated from the adjacent plate. Many columns toward the back in Fig. 2a are flat-topped mesas. One mesa has just connected with the opposite plate (middle “3” of the three in Fig. 2a), and the other two mesas labeled “3” have substantial overhangs on their upper face as well as shapes reminiscent of hyperboloid cooling towers. The bases of some of the columns (left-hand “3”) are angled considerably with respect to their tops. Figure 2b shows an area just to the right of that in Fig. 2a and shows similar structures. A column “oc” is visible on the obverse (opposite) side of this plate, and both images show significant plate curvature.

Figure 3 shows a slightly curved plate fragment from a second tooth and reveals a mix of the first three phases of column growth (labeled “1”–“3” and shown in Fig. 3b–d, respectively). Figure 3a views the fragment obliquely, and Fig. 3b–d show enlargements of columns 1–3 viewed nearly normal to the plate surface. The hemispherical deposit’s diameter is similar to those in Fig. 2, and its surface is relatively smooth at the magnification of Fig. 3b. At the same magnification, the nodules on the mound’s surface (Fig. 3c) are much rougher than the hemisphere surface and are somewhat smaller in size. The edge of the mound where it attaches to the underlying plate is a rounded rectangle similar to many mounds seen in Fig. 3a. The column in Fig. 3d has grown somewhat beyond those shown in Fig. 2, and its flat top indicates that it bridged the space between plates. Some fracture debris lies on the right side, and two cracks penetrate into the mesa from its flat surface.

A plate with gradient of column sizes is shown in Fig. 4a, and the flat mesa represent fourth phase columns (labeled 4 and 4’). Further growth proceeds laterally, and adjacent columns can merge (e.g. the two such columns 4’ in Fig. 4a). The columns have a pronounced directionality (i.e., the columns have visible major and minor axes and the major axes tend to lie along the same direction), and one of these columns (arrow in Fig. 4a) cantilevers from the surface while others appear to have fractured across on the same edge of the plate (upper left corner of the plate). Fragments of the lamella-needle complexes (LNC) are present on the right side of Fig. 4a. The area around the arrow in Fig. 4a was imaged at higher magnification (Fig. 4b), and the arrow in Fig. 4b shows where the cantilevered column *i* was displaced to *ii* by the electron beam while the rastered image was being collected. Comparison of Fig. 4a and b reveals columns *j* and *k* remain attached.

Figure 5 shows two plates split apart exposing the columns formed within the channel between the two plates. Panel (a) shows the entire plate fragments at low magnification, and the symbols define features shown at higher magnification in panels (b)–(d). Figure 5b shows part of the right-hand plate fragment where a mesa geometry column (at “m”, phase 3 of

column formation) and phase 4 columns (“4”) occur. Several small hemispheres (phase 1 columns) are also present (e.g. above “m”). On the left-hand fragment (Fig. 5c) mesa-shaped columns are prominent (%), and the plate has a glassy-type fracture surface in one direction and a cleavage type surface in the second. This cleavage surface is enlarged still further in Fig. 5d, and it is interesting that the cleavage face extends undeflected across a small, second phase column (#). There is a difference in contrast on the side of the plate midway between the two faces (its midplane).

Figure 6 compares elemental compositions for columns and the underlying plates for a third tooth. The micrograph in Fig. 6a shows the four areas where energy-dispersive x-ray spectra were recorded: Spectra 1 and 2 were collected on top of the mesa and 3 and 4 adjacent to it. Note that there are two small plates of material on the top of the mesa, and spectrum 2 was collected on one of these plates and 1 on the underlying column material. Spectra 1 and 2 were equivalent as were those at the pair of locations 3 and 4. Figure 6b shows the intensity of the Mg K-line was higher on top of the mesa than adjacent to it (just under 800 cts and ~500 cts, respectively). The simultaneously collected Ca K peak had spectra 3 and 4 greater than those of 1 and 2 (peak intensities of ~4,000 cts and ~3,500 cts, respectively). The ratios of the Ca and Mg peak intensities are typical of other mesas (phase 3 columns), nodular mounds (phase 2 columns) and hemispheres (phase 1 columns).

Structures observed on fracture surfaces may not reflect the in situ structure (i.e., within the intact tooth), but synchrotron microCT imaged column morphology within intact teeth. Figure 7a shows one wing of the flange of a *L. variegatus* tooth; second stage mineral formation is fairly advanced with phase 3 and 4 columns present. The 100×100 voxel area marked by the white box is enlarged in Fig. 7b, and the 3D rendering in Fig. 7c shows the same three columns as are seen in the enlarged area of the slice. These columns are just leaving the hyperboloid geometry and transitioning to a phase 4 geometry. These three columns roughly lie along a line perpendicular to the plates, i.e., they are collinear, and their positions are correlated between the adjacent channels.

Discussion

The micrographs of Fig. 2–5 show four phases of column growth. Figure 6 demonstrates that more Mg and less Ca are present in columns of the third developmental phase than in the underlying plate; this is typical both of more and of less developed columns. Some suggest that difference in the Mg:Ca ratio (columns vs plates) indicates a phase transformation from an amorphous precursor phase to a crystalline phase (20–22); on the other hand, crystalline material has been found in aboral plumula of living *L. variegatus* tooth (23) where advocates of amorphous precursors have insisted no crystalline material was to be found. These columns are an important part of tooth microstructure, first described as “soldering particles” over 150 years ago (6). The columnar interconnections between primary plates is a very primitive character dating back at least to the Carboniferous (> 300 Ma), see (24).

The mesa shaped column in Fig. 5c has a larger diameter where it attaches to the plate and smaller size on its free surface (where it fractured from the adjacent plate); this mesa has grown from the surface to which it remains attached. On the second plate fragment, i.e., on

the opposite side of the channel, small hemispheres are present on the plate surface between more developed columns (Fig. 5b). These micrographs show that columns grow into the channel from both plates bounding the syncytium, i.e., the situation in Fig. 1d,f applies and not that in Fig. 1c,e.

Figure 3 shows first and second phase columns are interspersed among third phase columns (mesas). First, the mixture of structures suggests that column nucleation is under very localized control. Second, the nearby presence of well-developed third phase columns does not prevent nucleation of new columns despite the competition for ions needed to form new very high Mg calcite. This is quite different from second phase growth in many inorganic systems where enlargement of pre-existing particles is favored over nucleation of new particles. Both the high and very high Mg calcite are thermodynamically unstable at ocean temperature, so it is not unreasonable to speculate that the macromolecules stabilizing growth of these non-equilibrium compositions may also allow new columns to grow adjacent to well-developed columns.

The large number of flat column tops exposed during fracture is consistent with the preferential fracture along the interface between column and plate, as has been described previously (12, 18). The earlier data, however, were on much more developed columns (phase 4 occupying most of the channel space between plates). When a mesa has just bridged a channel (phase 3) and the “base” has a significantly larger area of attachment to the underlying plate than the “apex”, it is not surprising that the mesa will fracture at the apical interface.

Fracture does not always follow the interface between the first and second stage mineral. In Fig. 4a, a column cantilevers from a plate although the electron beam was energetic enough to displace the column (Fig. 4b). In Fig. 5b, the same fracture continues from the underlying plate through the second phase column.

Drying the teeth does not introduce artifacts in the relatively mature volumes of the tooth imaged here by synchrotron microCT. The only changes we have observed in the mineralized structures of the tooth upon drying, occur in the most aboral portion of the intact shaft (where there are few connecting columns) and consist of cracks between rows of prisms within the keel.

Inferring the spatial distribution of columns on fractured plates is difficult because some can remain on the opposite plate or some can be lost entirely. MicroCT data (Fig. 7) show that the geometry of the columns exposed by fracture is consistent with those within intact teeth. The contrast within columns and plates is not reliable due to phase contrast effects and to missing mass in the local tomography imaging; contrast from differences in Mg content is lower than that of these other effects. Nonetheless, the reconstructed surfaces of the columns and plates are reliable, as expected for local tomography (25).

MicroCT of intact teeth allows spatial correlation of columns in different channels to be probed. Many of the columns like those in Fig. 7b,c are roughly collinear; others, not shown, are not. We speculate that columns form at very specific channel positions relative to the syncytium’s nuclei, but testing this hypothesis would be very difficult as it is an intrinsically

3D problem and not amenable to serial sectioning and nuclear staining. One could perform submicrometer synchrotron microCT at even higher resolution than in the present case, and the first through third phases columns would be visible (i.e., higher resolution than in Fig. 7 and at a more aboral, less developed portion of the tooth). If the labeling were possible with nuclei-specific metal stains, microCT could also map the nuclei spatial distribution by, for example, imaging the tooth with photon energies above and below the metal's K- or L-edge, where only voxels containing the metal would show significant changes in contrast, e.g. (26).

The spatial distribution of columns in the microCT data has not been quantified here because the column density is rather large in the portion of the tooth imaged. Examining the distribution of nucleation sites would require imaging a much less mature section of the tooth. Some initial data have been reported elsewhere, however, about the rate of column growth using synchrotron microCT with voxels $> 1 \mu\text{m}$ in size (16). Quantifying shape with microCT requires voxel sizes like those used here and imaging of earlier stages of column formation than are shown here.

The curvature of the plate shown in Fig. 2 is consistent only with a secondary plate that curves toward the transition with the carinar process plates. In *L. variegatus*, the primary plates do not show this curvature, and the carinar process plates are wavy. The non-parallel top and bottom of some mesas (e.g. left hand “3” in Fig. 2a) are consistent with the secondary plate identification. None of the plates in Fig. 3–5 are wavy, so they cannot be carinar process plates. The plate in Fig. 3 shows some curvature but is small enough that it is difficult to know whether it was a primary or secondary plate, and the same is true of the fragments in Fig. 5. The long flat plates in Fig. 4 are associated with fragments of the lamellar needle complex and this indicates that they are primary plates.

As columns linking adjacent first stage mineral structure appear to be a tooth character of the basal echinoid clade (24), one expects columns to develop in the same fashion within Toxopneustidae, in other families of the order and in the other orders. Geometry of the channels between first stage mineral structures may affect how the columns develop. For example, the teeth of *Micropygus*, *Phormosoma* and *Tromikosoma* have very different and more open structures than *Lytechinus* (27). Prisms within the keels of camarodonts are likewise joined by second stage mineral, and the same phases of column development should be present although cementing the curving surfaces of prisms may impose differences compared to plates.

Figure 6 shows higher Mg content in a small flat-topped column than in an adjacent position of the underlying plate. The intensity of the Mg peak (less background) is proportional to the number of Mg atoms present in the sampled volume, and the ratio of Mg intensity from the column to that of the nearby plate, i.e., $I_{\text{column}}/I_{\text{plate}}$ is 1.6–1.7 for the column in Fig. 6. A second column of about the same size has $I_{\text{column}}/I_{\text{plate}} \sim 1.8$ –1.9, and a position closer to the edge than the center of a larger diameter column has $I_{\text{column}}/I_{\text{plate}} \sim 2.9$. Note that, in all three cases, the column and plate surfaces are flat and parallel, and effects from differential geometry will be absent. Based upon precision lattice parameter determinations from position-resolved synchrotron x-ray diffraction and calibration curves (Mg content vs lattice

parameter), one expects Mg mole fractions in the second stage mineral to be 0.22 to 0.29 higher than in the first stage mineral (15); this corresponds to ratios between 2.5 and 2.9 for Mg in columns relative to plates.

The higher Mg content in the larger column may suggest that calcite added to the perimeter of a column incorporates more Mg than is initially present. Earlier, secondary ion mass spectroscopy (SIMS) mapping of *L. variegatus* tooth cross-sections did not reveal variable Mg content within columns (28), but others reported variable Mg content along the length of a prism dissected from a tooth (12). The focus here is not the Mg content of columns but rather the identification of the structures seen in the SEM as the same structures as are seen in microCT. Much more data would be needed on the column vs plate Mg composition for teeth of *L. variegatus* and of other taxa before one could arrive at firm conclusions about composition and its micrometer-level variation. In addition to SEM or SIMS, position resolved x-ray diffraction or x-ray excited x-ray fluorescence with sub-micrometer diameter beams might be profitably employed on thin samples, e.g. studies mapping peritubular vs intertubular dentin (29).

The role of Mg in growth of sea urchin calcite remains to be established, and the authors speculate that Mg could speed mineralization, whether by enabling formation of a transient amorphous precursor phase or by increasing the cations available for incorporation in the crystal lattice (e.g. (30)). At the most active stages of second stage (column) formation in the *L. variegatus* tooth flange, the rate of mineral addition is quite large: Stock (16) estimated $\sim 1.2 \times 10^4 \mu\text{m}^3$ of very high Mg calcite was added to a volume of $270 \times 270 \times 1.45 \mu\text{m}^3$ within 1.7 days. As Cavey and Märkel noted (31), ions for forming second stage mineral must travel in channels between impervious plates, distances up to $\sim 300 \mu\text{m}$ (16), and this, together with the amount of material formed and the restricted width of the channels, demonstrates that fluxes of Ca and Mg ions must be very large. Thus, the urchin may have evolved a tooth mineralization system making use all of all available divalent cations.

The present study does not examine the relationship between column and plate crystallography. Earlier, x-ray microbeam diffraction of teeth from regular sea urchins spanning the full phylogenic range showed that first stage and second stage mineral invariably has the same crystallographic orientation within the sampled volumes (15); this includes orders (families) Cidaroida (Cidaridae), Arbacioida (Arbaciidae), Temnopleuroida (Temnopleuridae) and Echinoida (Toxopneustidae, Echinometridae, Strongylocentrotidae and Parechinidae). These diffraction studies (as well as birefringence studies (3)) demonstrate co-orientation of column and plate, at the least, and are consistent with epitaxy of the two structures. The techniques used in the present study, microCT and SEM did not provide adequate spatial resolution to prove or disprove the presence of epitaxy. An additional complication is the presence of the organic layer on the surface of the plates and between column and plate (28) and whether this layer has gaps or not. Lattice fringe imaging with transmission electron microscopy (TEM) or atomic force microscopy would, for example, be required to establish whether the interface was coherent or partially coherent or whether the column and plate were in contact at all. The authors note that TEM of sea urchin teeth is challenging because of the material's susceptibility to beam damage (32). X-ray diffraction with submicrometer diameter beams and thinner specimens might also show

whether small misorientations exist between column and bounding plates, misorientations that were too small to detect in earlier studies.

The data of the present study demonstrate that the columns grow along the underlying plates. The authors suspect that the columns nucleate heterogeneously, but testing this hypothesis would require flash freezing of a living tooth and cryo TEM imaging or nanoCT of a living or (flash) frozen tooth. Four stages of column microstructures are observed on fractured plates, and all of these developmental phases can be seen on the same plate within the span of a few tens of micrometers. This suggests column nucleation is under very localized control. Columns nucleate and grow on both sides of the channel between adjacent plates. Synchrotron microComputed Tomography (microCT) with 280 nm isotropic volume elements (voxels) demonstrates that the structures seen on fractured plates are present within the undamaged *L. variegatus* tooth.

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Figure 1.

Schematic of growth of first (plates, light gray) and second stage (columns, dark gray) mineral. Nuclei are shown as solid black ellipses, and the syncytial walls are also shown in black. (a) Plates (first stage mineral) growing in syncytial spaces. The light gray arrowheads show the direction of plate growth. (b) Stage after plate growth is complete but before columns (second stage mineral) start to grow. (c and e) Growth from the plate on one side of the syncytium. (d and f) Growth from both plates bordering the syncytium. The structures in (c) and (d) represent the first phase of column formation and those in (e) and (f) the second

phase. (g) Columns just connecting the plates (third phase of column growth). (h) Columns expanding laterally (fourth phase).

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Figure 2.

First three phases of column growth in *L. variegatus* imaged with secondary electrons in SEM. (a) Hemispheres, mounds and mesas exposed during fracture (first “1”, second “2” and third “3” phase columns, respectively). (b) Edge of the same plate showing columns “oc” on the opposite plate face.

Figure 3.

A second *L. variegatus* plate fragment viewed with secondary electrons. (a) The entire fragment with columns at the three phases of development labeled "1"–"3", respectively. (b–d) show enlargements of columns "1"–"3", respectively.

Figure 4.

Secondary electron images of a third *L. variegatus* plate fragment. (a) A gradient of column sizes with fourth phase structures labeled "4" and fragments of lamella-needle complexes "LNC". Column 4' shows merged columns, and the horizontal arrow indicated the area enlarged in the second panel. (b) Enlarged area showing the column jutting over the edge of the plate "i" displaced by the electron beam to "ii" while plates "j" and "k" did not move.

Figure 5.

Secondary electron images of a pair of plates split apart by fracture. (a) Lower magnification view shows both plates. The labels “m”, “%” and “#” locate the features enlarged in (b-d). (b) Mesa-shaped column (phase 3) labeled “m” and several phase 4 columns around “4”. (c) Mesa-shaped column “%”. (d) Second stage column “#” cleaved along the same plane as the underlying primary plate. The inset at the lower left of this panel shows the column with contrast stretched to make the column-plate interface visible in the figure.

Figure 6. Energy dispersive x-ray spectroscopy of top of an exposed mesa and of the adjacent plate surface. (a) Secondary electron micrograph showing the four areas where the spectra were recorded. (b) Intensity vs energy of the Mg K line. (c) Intensity vs energy of the Ca K line.

Figure 7.

Synchrotron microCT of one wing of the flange of a *L. variegatus* tooth. (a) Slice with stone part “S”, secondary plates “SP” and primary plates “PP” labeled. The vertical field of view extends 2048 voxels (voxel size is 280 nm). The white square shows the area enlarged in the upper right panel. (b) Three columns in three adjacent channels in the 100×100 voxel enlargement. (c) 3D rendering of the columns in the upper right panel. The bottom-most column is only partially visible.