Preservation of *Triceratops horridus* Tissue Cells from the Hell Creek Formation, MT

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Abstract: Dinosaur soft tissues are shown to be remarkably preserved to the sub-micron level of ultrastructure despite environmental and biological factors associated with burial for millions of years. Light microscopy and scanning electron microscopy (SEM) reveals soft tissue features such as fibrillar bone tissue, osteocytes, and blood vessels. Concerns that these findings relate to contamination or biofilm formation have been refuted. Notwithstanding the controversial nature of these discoveries, soft dinosaur tissues should be systematically searched for and thoroughly characterized in other dinosaur remains.

Introduction

Remarkably preserved cells and tissues from dinosaurs have been reported since the mid 1960s [1], however until recently, dinosaur bone specimens usually have not been decalcified or otherwise destructively studied for the presence of soft tissues because complete bone specimens are highly prized by paleontologists and collectors. Over the past 50 years, soft blood vessels, collagen bands, intact cells, bone cells (osteocytes), filopodia with primary and secondary branching, cell-to-cell junctions, intracellular nuclei, and other soft tissue details have been observed and illustrated from various different species of dinosaurs including *Tarbosaurus bataar*, *Tyrannosaurus rex*, *Brachylophosaurus canadensis*, and *Triceratops horridus*. [1–6]. Initial criticisms, which labeled these soft structures as biofilms [6], have been resolved as incorrect [7].

In 2012 I collected a large *Triceratops horridus* supraorbital horn from the Hell Creek Formation at Glendive, Montana. The horn yielded soft sheets of fibrillar bone (Figure 1) and life-like cells. A *Triceratops* rib specimen from the same deposit contained soft blood vessels and red blood cell-like (RBC) microstructures. Remarkable preservation of individual bone osteocytes encapsulated within the stretchy sheets of fibrillar horn bone was observed, as were osteocytes positioned upon sheets of fibrillar bone adhering to permineralized vessels within the decalcified horn bone [6]. Variable-pressure scanning electron microscopy (VPSEM) of uncoated specimens was not attempted at that time, nor were individual cells isolated from the specimen for further analysis. In this article I describe VPSEM and cell isolation results from the *Triceratops* horn.

Materials and Methods

Specimen preparation. The hand-sized pieces of the horn, somewhat "pie-slice" in shape and extending from the exterior horn surface to the inner trabecular (cancellous) bone (core), were fixed in a 2.5% solution of glutaraldehyde, buffered with 0.1 M sodium cacodylate buffer at 4°C for 5 days, rinsed in distilled water and buffer, and stored in phosphate buffered saline (PBS). Pieces, roughly 20 mm^3 in size, were extracted from the inner bone core by pressure fracture and were processed through a decalcification protocol as follows: bone pieces were rinsed in pure water after fixation and were incubated in a solution of 14% sodium ethylenediamine tetraacetic acid (EDTA) at room temperature. The EDTA was exchanged every 2 to 4 days for a period of 4 weeks after which bone fragments were processed for scanning electron microscopy (SEM).

Other pieces were soaked for 4 months in EDTA. Even after this treatment significant bone mineral/hardened material remained; therefore, it is unknown whether complete decalcification in EDTA would yield soft and transparent, vessel-like tissues, such as previously reported [7–11]. A soak in hydrofluoric acid (HF) was not attempted, but it might prove more successful in liberating any soft vessels that remain. Rib specimens were similarly fixed, washed, and pressure fractured to reveal inner surfaces of compact bone (Figures 2 and 3).

Light microscopy of cells. Aliquots of decalcification solutions (post soak) were transferred by pipette into tied off chambers of *Snakeskin* dialysis tubing, (Thermo Scientific, Rockford, IL) and were submerged into vials of distilled water for 2 weeks. Water was exchanged every 2 days, and after 2 weeks cells were transferred after dialysis onto glass microscope slides for examination and imaging on a Jenaval light microscope (Carl Zeiss Jena) equipped with a Jenoptik ProgRes (Jena, Germany) $C14^{plus}$ camera.

SEM imaging of bone. After a 4-week soak in EDTA, decalcified bone was air-dried and affixed to aluminum stubs. For Figures 2–4, bone specimens were sputter-coated with

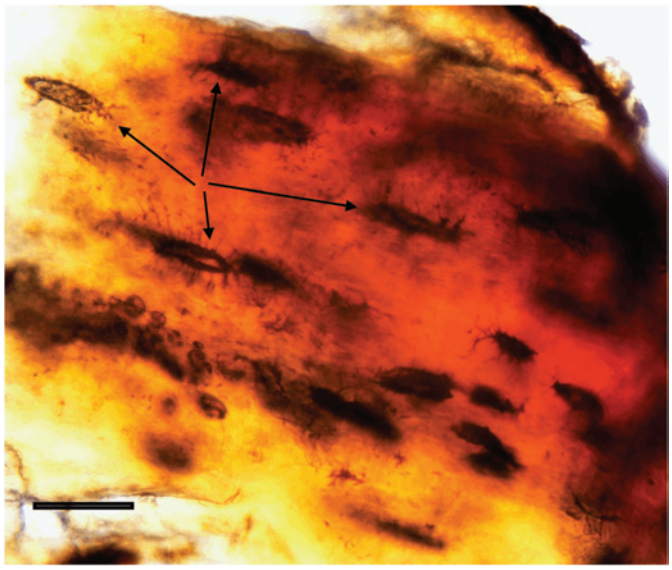

Figure 1: Portion of soft, stretchy fibrillar bone from *Triceratops* horn. Note embedded osteocytes (black arrows). Scale bar = 30 µm.

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SEEING the BIG PICTURE

How did your role as Sustainable Laboratories Coordinator lead to an interest in the field of microscopy?

I work with the sustainability aspects of scientific research, particularly the facility costs (water, electricity, chemicals, etc.). Microscopes are essential research tools and so most facilities I work with own and use them. I was curious as to what opportunities for efficiencies or cost savings there might be.

What are the challenges in driving adoption of modern efficient lighting technologies?

The overall challenge is always convincing users that the hardware can deliver for a price that makes sense.

"Illumination for research-grade microscopy presents the particular challenge of having a wide range of technical requirements (wavelength, power, spatial and temporal distribution)."

From my side, I first wanted to know that users were happy with the technical features of Lumencor light engines. I can't promote something they don't actually want. After organizing the purchase of 5 Lumencor light engines for the University of Edinburgh I'm happy to report I've heard nothing but praise for the new systems.

How have Lumencor's products addressed these challenges?

From the feedback of researchers, Lumencor manufactures some of the most powerful solid-state illumination systems available. These systems may entail a significant purchase-cost increase when compared to mercury-arc systems. But in the big picture, whole-life costing of the systems produces a different conclusion.

"When bulb costs and unproductive idle time are factored in, purchasing a solid-state light source can save £8,000–£25,000 ($12,000–$40,000) over its projected working life compared to mercury-arc sources."

This doesn't factor in mechanical cooling savings from reduced heat output of Lumencor light engines, which would increase savings. Importantly the bulk of these savings go back to the researcher, as they pay for bulb costs. At the University of Edinburgh, we purchased 5 Lumencor light engines instead of mercury-arc lamps, saving researchers a minimum of £40,000 ($60,000) over the equipment lifetime, while at the same time eliminating the repetitive tasks of mercury bulb replacement and disposal.

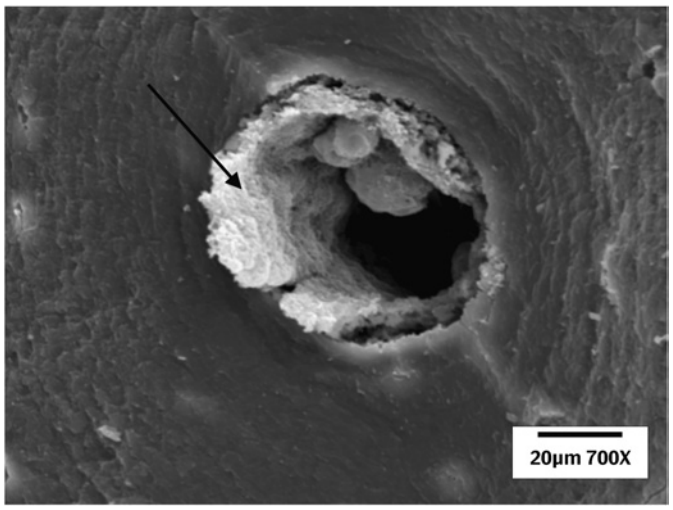

Figure 2: Soft blood vessel wall extending from Haversian canal (black arrow), *Triceratops* rib. Scale bar = 20 µm.

gold for 90 seconds at 20 mA and were imaged at 30 kV under high vacuum in a Hitachi S2500 SEM. Bone specimens in Figures 5–6 were left uncoated and imaged at 10 kV in a Zeiss EVO SEM with a backscatter electron detector. Bones in Figures 7 and 8 were left uncoated by metal and imaged in a Zeiss EVO SEM at 3.1×10^{-4} Pa with a secondary electron detector.

Results

Fibrillar Bone. Soft, stretchy sheets of fibrillar bone tissue exhibited layers of bone cells (osteocytes). These would come into focus depending on the layer imaged in light microscopy (Figure 1).

Blood vessels. Soft blood vessels (arrow in Figure 2) extended from many Haversian canals in the fractured rib. It is clear that when the living vessel was fully extended by blood and serum, it tightly abutted against the undulations in the Haversian canal wall. Spherical microstructures, consistent with the size and shape of RBCs, were observed in the lumens of many vessels in non-decalcified *Triceratops* rib specimens

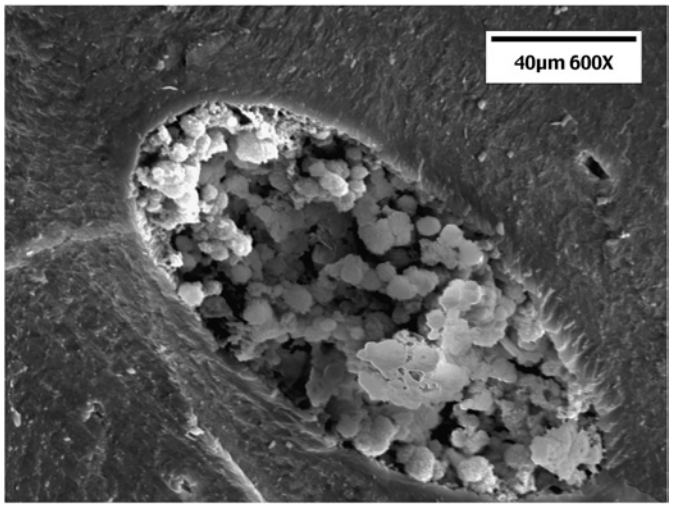

Figure 3: Spherical microstructures, consistent with size of RBCs, within Haversian canal of *Triceratops* rib. Scale bar = 40 µm.

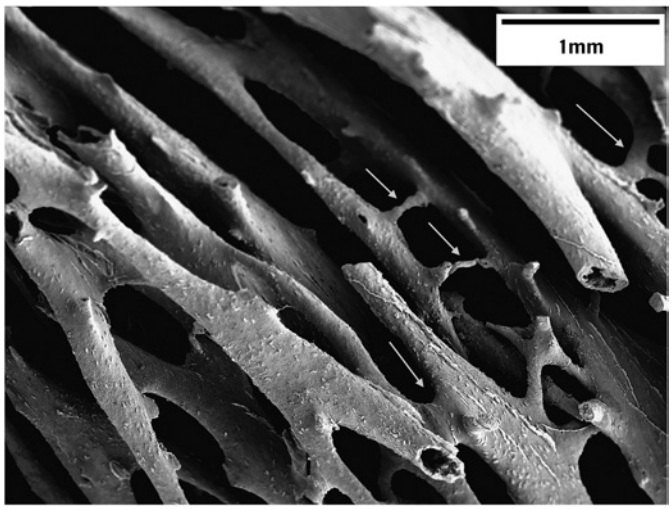

Figure 4: Blood vessels from decalcified sample of *Triceratops* horn. Cross-linking Volkman's canals are evident (white arrows). It was on the surfaces of these blood vessels where soft bone osteocytes were found (discrete dots on vessels). Scale bar = 1 mm.

(Figure 3). The amount of rib material collected did not allow for decalcification experiments, therefore it is unknown if large regions of soft vessels within the rib were preserved.

Horn blood vessels appeared to be permineralized after decalcification removed the bone mineral (Figure 4). Volkmann canals were present (Figure 4 arrows), and the outside surfaces of vessels were wrapped in soft fibrillar bone sheets. Figures 4 and 5 show many osteocytes on the surface of these sheets as well as within adjoining sheets of fibrillar bone (small white dots on the surfaces of vessels, in Figure 4 and arrows on Figure 5).

Bone cells. Osteocytes imaged with SEM exhibited smoothly tapered filopodia, which extended to 20 µm in length in some instances (white arrows in Figures 6 and 7). A band of preserved collagen is labeled with a black arrow in Figure 7.

Discussion

The remarkable preservation of delicate ultrastructures such as filopodia and cell-to-cell junctions (white arrows, Figures 6 and 7) has resisted a simple explanation despite hypothesized

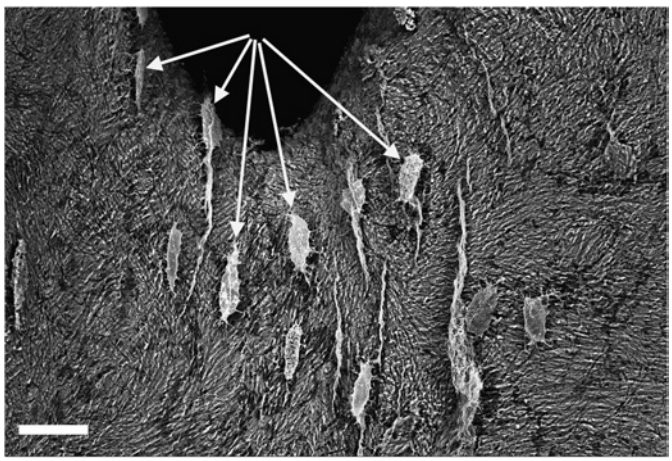

Figure 5: Numerous soft bone osteocytes are seen on the surface of a blood vessel (white arrows). Scale bar = 35 µm.

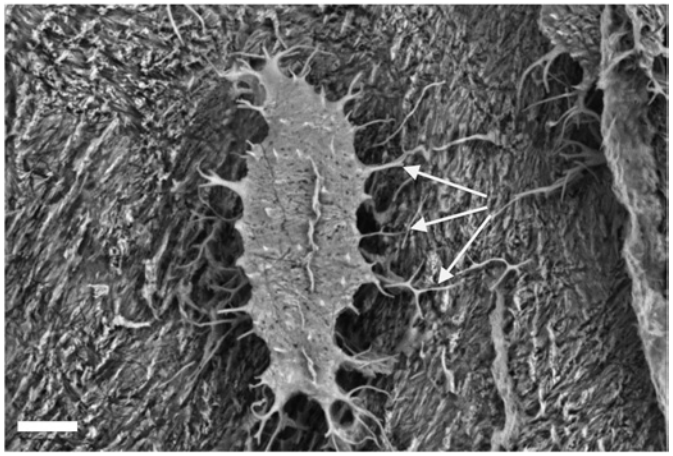

Figure 6: A single soft osteocyte from *Triceratops* horn. Delicate filipodia extend out to connect with other cells (white arrows). Scale bar = 4 µm.

temporal limits on molecular preservation over millions of years [13]. In the case of soft vessels recovered from dinosaur femur specimens, it seems reasonable that these tissues were sequestered from the elements and from biological scavenging activity because of deep encapsulation within compact bone. Within the *Triceratops* horn, however, which was highly vascular, no sequestration was likely because all of the vessels were openly exposed to air, soil, water, scavengers, dissolved salts and minerals, and the freeze-thaw cycle and heat of Montana seasonal weather; yet a high degree of preservation persists. While plant roots, fungal hyphae, and insect remains were all found traversing the horn, soft fibrillar sheets of bone and well-preserved osteocytes remain.

Discoveries of soft blood vessels, RBC-like microstructures, and soft bone osteocytes have been controversial [8–10]. One criticism maintained that these soft tissue discoveries are not endogenous tissues but rather the remains of bacterial biofilms, which "retain much of the original morphology" of dinosaur bone osteocytes and filipodia [8]. However osteocytes from other dinosaur specimens were later demonstrated to contain actin, tubulin, and histone H4 proteins, which are not found in bacteria or bacterial biofilms. These osteocyte proteins are consistent with other dinosaur protein finds [7].

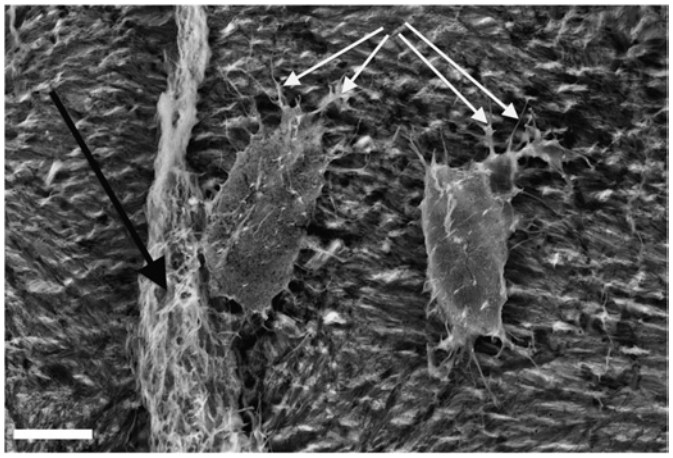

Figure 7: Fine structure filipodia extend as dendrites from two soft osteocytes in *Triceratops* horn (white arrows). Black arrow is pointing to a band of collagen. Scale bar = 10 µm.

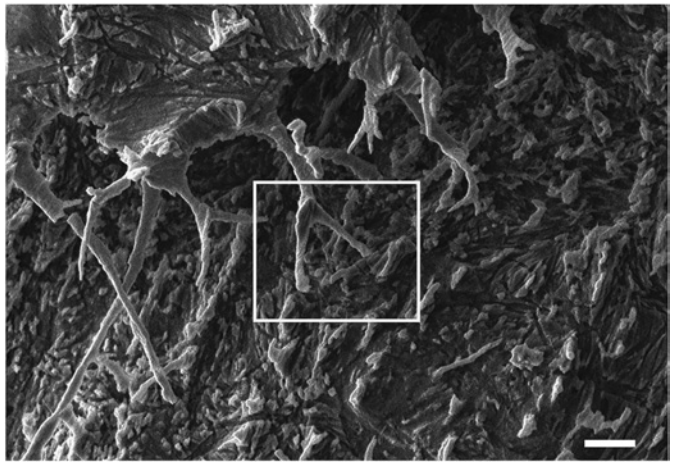

Figure 8: White box surrounding delicate bifurcated termination of filipodia. Smooth edges of dendrite indicates the high degree of preservation. Scale bar = 7 µm.

Uncoated specimens of decalcified bone in this study yielded osteocytes with a higher degree of ultrastructural preservation than previously reported [3,6,9,12]. Uncoated bone surfaces show lacunae depressions (Figures 5–7), extensive filopodia (Figures 6–10), collagen aggregates (Figure 7), and cell surfaces displaying the indented impressions of overlying and compressing bone (Figures 6 and 8).

Figures 9 and 10 show cells that were successfully isolated from fibrillar bone. In future work, it is hoped that individual cells such as these can be examined using immunohistochemistry for the presence of endogenous proteins.

Conclusion

Claims of contamination and biofilm replication have been dismissed [7,12], and identification of intra-cellular and intra-nuclear proteins have been verified showing that these are endogenous dinosaur tissues [12]. Therefore claims that these are not original dinosaur tissues appear to be questionable.

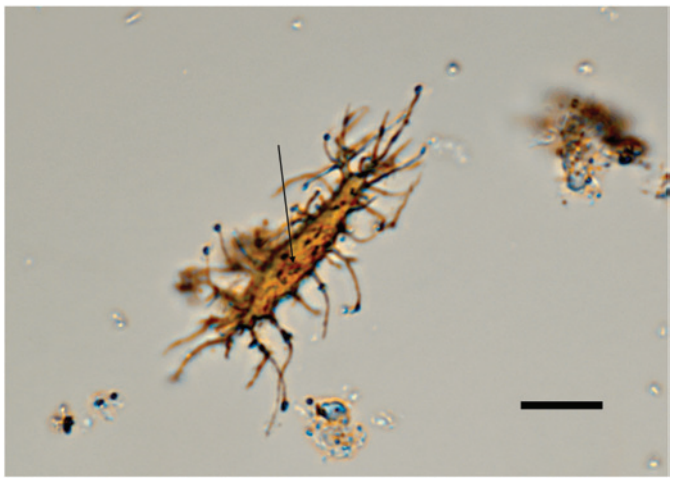

Figure 9: Isolated and washed *Triceratops* soft bone osteocyte under light microscopy. Cells often contained nucleus-like microstructures (black arrow). Scale bar = 5 µm.

Figure 10: Isolated and washed *Triceratops* soft bone osteocyte under light microscopy. Note many elongated filipodia. Scale bar = 8 µm.

Original dinosaur soft tissues are shown here to be remarkably preserved to the sub-micron level of ultrastructure despite the environmental and biological factors associated with its burial. Notwithstanding the controversial nature of these discoveries, soft dinosaur tissues should be systematically searched for and thoroughly characterized in other dinosaur remains.

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