Cross-Polarisation and Second Harmonic Generation Imaging Reveal Bone Collagen Decay Patterns in Four Fossils

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DETECTING COLLAGEN DECAY

A steady stream of publications show morphological and molecular signs of protein remnants in fossils across geologic time and geographic space, as our recent review documented.[1] Protein kinetics imply a much shorter shelf-life than standard fossil ages permit. This incongruity in timing remains unresolved. New approaches are needed to reveal biochemical decay patterns relevant to this research question.

Polarized Light Microscopy

A polarizing light microscope equipped with a first order red retardation plate reveals pleochroism, which occurs when components of a divided light beam follow different paths and travel at different speeds through a crystalline substance. Rotating the stage changes the angles and the resulting colors in the same regions of pleochroic minerals and collagenated bone.[2]

Fig. 1. Zeiss Photomicroscope with 1st order red filter and Panasonic Lumix S1 digital full frame camera

Second-Harmonic Generation Imaging

SHG confocal microscopy uses a 920nm laser to target type I collagen fibres in bone.[3] Typically used on live tissue for biomedicine, our prior work demonstrated that SHG reveals decayed collagenous structures in ancient, subfossil, and fossil bone.[4] SHG occurs when two excitation photons interact with densely packed, parallel amide bonds such as in collagen.

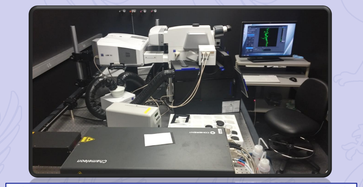

Fig. 2. Zeiss Examiner Z1 two-photon excitation laser scanning confocal microscope coupled to a Coherent Chameleon titanium: sapphire laser at UT Southwestern Medical Center Live Cell Imaging facility.

CROSS-POLARIZED LIGHT

Modern Versus Fossil

Densely collagenated modern bone (*H. sapiens* MSSC.R 3-8) was thinsectioned and mounted for a cross-polarized light microscopy (XPOL) comparison with fossil bone (Permian *Diadectes sp.* MSSC.P 19-1). Figure 3A shows gold and blue collagen birefringence in modern bone with a 1st order red filter (XPOL-R). Pleochroic gold and blue bands suggest decayed collagen in *Diadectes*.

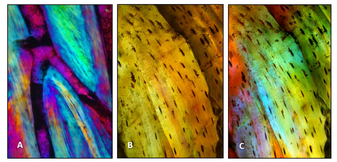

Fig. 3. Birefringence in *Diadectes sp.* vertebra and modern *Homo sapiens* bone reveals collagenous signal A) XPOL with 2nd order red filter reveals strong gold, blue and lavender birefringence consistent with dense collagen B) *Diadectes* vertebra shows general bone architecture. C). XPOL-R reveals birefringences consistent with diminished collagen. Scale bar 50um.

Cretaceous

Edmontosaurus annectens (MSSC.J 12-7) spongy bone thinsection reveals a mixture of non-collagenated and faintly-collagenated bone microregions. XPOL micrographs appear to expose "dead" bone regions that do not change color under rotated cross-polars.

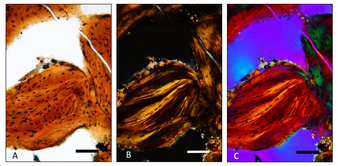

Fig. 4. *Edmontosaurus* thinsections reveal possible collagenous traces. A) Brightfield (BF) image shows general bone structure. B) XPOL with cross polars extinguished shows strands of birefringence. C) XPOL-R reveals pockets (green and gold) of birefringence beside strands of non-collagenated bone. Scale bar 50um.

Jurassic

The same sequence of photography used in Fig. 4 was applied to a plesiosaurid vertebra (MSSC.C 13-20) from Isle of Wight, UK. Fragmented bone with partial permineralisation indicate diagenetic alteration. Brightly colored angular shapes in Figure 5B,C represent calcite and permineralisation. Figure 5B,C show a small region of mild, birefringence with diffuse margins, consistent with highly degraded collagenous remnants.

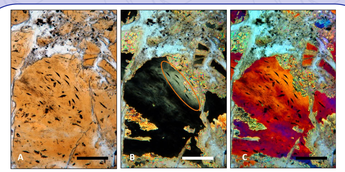

Fig. 5. Plesiosaurid thinsections reveal bone degradation. A) BF image shows general bone structure intact in central region. B) XPOL with cross polars shows faint band (oval) of birefringence, with most of the region "collagen dead." Bright yellows represent permineralisation. C) XPOL-R confirms faint birefringence in region of interest. Scale 50um.

Permian

Eryops megacephalus (MSSC-P16-11), limb bone thinsection reveals a mixture of non-collagenated and faintly-collagenated bone microregions. Figure 6B-D show strong birefringence in concentric bands that wrap around some canals.

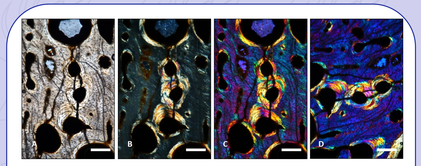

Fig. 6. *Eryops* thinsections reveal birefringence near to canals, indicative of collagenous traces. A) BF image shows general bone structure with diagenetic cracking. B) XPOL with cross polars shows rings of birefringence. C) XPOL-R confirms rings. D) . XPOL-R and stage rotated 90° reveals shifting colors when compared to (C), also consistent with collagen. Scale bar 50um.

SECOND-HARMONIC GENERATION

Permian

In our prior work, SHG imaging was applied to archaeological and fossil bones.[4,5] This technique was extended to *Eryops* (MSSC. P 15-8) skull material here. SHG signal occurred in striated blotches, small patches, and on osteonal linings. Figure 7A shows SHG in "fire," which assigns the whitest color to the brightest pixels. SHG patterns appear consistent with collagen remnants. Independent verification could confirm this outcome.

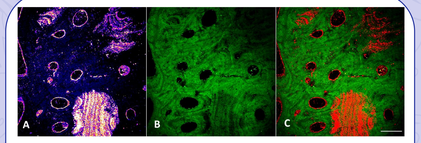

Fig. 7. SHG imaging of Eryops skull. A) SHG signal rendered as "fire" in ImageJ, B) Autofluorescence collected at 760nm reflects organics and shows bone context. C) SHG rendered red and autofluorescence composite shows banded SHG signals in bone and SHG rings as osteonal lining. Brightness range 800-2000, scale bar 200um.

Mineral Reflectance Test

To test if the SHG signal in endosteal walls as seen in Figure 7 represents laser reflectance off minerals, epi-illumination micrographs were collected from areas overlapping SHG. Results show calcite infill and probable iron oxides that overlap part of the SHG signal, plus SHG signal where no minerals are.

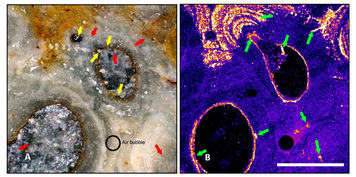

Fig. 8. Epi-illumination and SHG comparison. A) Epi-illumination shows unique features. Yellow arrows signal structures seen in epi- only. Red arrows show sites of SHG features absent from epi-.B) SHG of same area. Green arrows indicate SHG features with no corresponding structures in epi-illumination.

CONCLUSIONS

XPOL and XPOL-R micrographs present pleochroism in bone thinsections from Permian, Jurassic, Cretaceous, and modern sources. Regions where gold bands turn blue and vice versa when the stage is rotated under XPOL-R are consistent with collagenous remnants in fossil bone. Whereas bright colors under XPOL of modern bone show dense collagen throughout, less bright colors show diminished collagen signal, likely from collagen decay.

Decay patterns include collagen probably retreating into shrinking zones. Collagen-caused birefringence can occur regardless of permineralisation. Geologic settings do not affect either the apparent presence of collagen nor its decay pattern in our sample set.

SHG images reveal possible collagen in small patches. Some SHG signal may overlap iron mineralisation, for example in osteonal linings. Alternatively, osteonal lining could include residual epithelial tissue. Additional work will be required to test the lining material constituents and to directly cross-examine XPOL and SHG as techniques with potential to investigate collagen decay patterns in fossil bone.

References

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3. Ambekar, R. et al. *Bone*. 50(2012): 643-650, 2012.
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Abbreviations key:

MSSC = Microscopical Society of Southern California
BF = Brightfield microscopy
SHG = Second-Harmonic Generation imaging
XPOL = Cross-Polarized Light microscopy
XPOL-R = Cross-Polarized Light with 2nd Order Red filter
Epi = Epifluorescence, or reflected light, illumination

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