



### **A86 Optimization of a Protocol for Visualizing Vascular and Cellular Pore Networks in Human Bone Using Multiphoton Confocal Microscopy**

*Mary E. Cole, MA\*, Ohio State University, Dept of Anthropology, 4034 Smith Laboratory, 174 W 18th Avenue, Columbus, OH 43210-1106; and Sam D. Stout, PhD, Ohio State University, Dept of Anthropology, 4034 Smith Laboratory, Columbus, OH 43210-1106*

After attending this presentation, attendees will appreciate the potential of multiphoton confocal microscopy for 3D visualization of human bone loss at the histological level.

This presentation will impact the forensic science community by optimizing a protocol for 3D imaging of vascular and cellular spaces in bone tissue. Future forensic applications for imaging these pore structures include: (1) identifying bone tissue sites at high risk for spontaneous fracture; (2) distinguishing between different bone types at the tissue level based on pore geometry, which appears highly sensitive to mechanical loading environment; and, (3) demonstrating the advantages of multiphoton confocal microscopy for histomorphological analysis in general.

Throughout life, bone is remodeled to replace old tissue, repair microscopic damage, or resorb bone in response to decreasing mechanical strain. Osteoclasts tunnel into the old bone, and osteoblasts fill this resorption space with new bone, leaving a central Haversian canal to transmit a blood vessel. This “vascular porosity” increases with age as osteoblasts decline in their capacity for bone formation, while osteoclasts increasingly resorb bone in response to declining physical activity and muscle strength. Vascular pores are stress concentrators for microdamage, which can initiate and propagate into a spontaneous fracture within the highly interconnected pore network. Bone tissue also has a separate network of “cellular porosity,” composed of the lacunae that house osteocyte cells and their connecting canaliculi. Porosity shows promise for distinguishing between bone regions and types based on mechanical loading environment. Regions of lower mechanical strain within a given bone have higher vascular porosity. Loaded bones have been found to have larger lacunar volumes and more branched canaliculi compared to the unloaded bones.

3D pore geometry is poorly characterized. Forensic scientists interested in associating pore geometry with different bone types (e.g., normal/osteoporotic, young/old, human/non-human, loaded/unloaded) need an effective protocol for imaging these pore structures. Ciani et al. stained rat tibiae with Fluorescein Isothiocyanate Isomer I (FITC) and imaged cellular pore networks with single-photon confocal laser scanning microscopy.<sup>1</sup>

The suitability of this protocol was tested for imaging both vascular and cellular pore networks in human bone. Multiphoton confocal microscopy was used as it can penetrate far deeper into a sample with infrared light. Ten cross sections, approximately 500µm to 600µm in thickness, were cut from the midshaft of a fresh cadaveric human rib. Each cross section was placed in a 15mL volume of freshly prepared 4% formaldehyde and fixed for 24 hours at room temperature under gentle rotation. Cross sections were dehydrated in ascending grade ethanol (75%, 95%, and 100% for five minutes each). FITC was diluted in 100% ethanol at concentrations of 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.2%, and 0.1%. Each 10mL solution was gently rotated for one hour, then filtered. Each cross section was placed in a different concentration of solution for four hours under gentle rotation. Each cross section was then rinsed in 15mL of 100% ethanol under gentle rotation for 30 minutes, then air dried. Small regions were imaged using an Olympus® FV1000 MPE Multiphoton Laser Scanning Confocal microscope with N.A. 1.05, 25x water immersion objective, 800nm laser excitation wavelength, resolution 1,024 x 1,024 pixels, step size 0.63µm,



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and 2x Kalman averaging. 3D vascular and cellular pore networks were analyzed for volume, orientation, and connectivity in the image analysis software FIJI.

Results were best for a concentration of 1% FITC, as resolution was lost as most features became indistinguishable at a depth of approximately 185 $\mu$ m into the sample. Vascular and cellular pores were clearest to a depth of approximately 100 $\mu$ m. Pores displayed fairly uniform brightness within a given depth. At concentrations of FITC <1%, the resolvable depth decreased, and pore structures became more variable in brightness within a given depth.

These results demonstrate that the method from Ciani et al. is suitable for 3D imaging of both vascular and cellular pore networks in human bone tissue. Multiphoton confocal microscopy facilitates deep penetrate into the sample to allow quantification of pore volume, orientation, and connectivity.

### Reference(s):

1. Ciani C., Doty S.B., Fritton S.P. 2009. An effective histological staining process to visualize bone interstitial fluid space using confocal microscopy. *Bone*. 44: 1015 – 1017.

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### Skeletal Histology, Confocal Microscopy, 3D Imaging