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Collagen orientation patterns in human secondary osteons, quantified in the radial direction by confocal microscopy

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Abstract

The composite structure of secondary osteon lamellae, key micro-mechanical components of human bone, has intrigued researchers for the last 300 years. Scanning confocal microscopy here for the first time systematically quantifies collagen orientations by location within the lamellar thickness. Fully calcified lamellar specimens, extinct or bright in cross-section under circularly polarized light, were gently flattened, and then examined along their thickness direction, the radial direction in the previously embedding osteon. Collagen orientation was measured from confocal image stacks. So-called extinct lamellae and so-called bright lamellae are found to display distinct, characteristic patterns of collagen orientation distribution. Orientations longitudinal to the osteon axis in extinct lamellae, transverse to the osteon axis in bright lamellae, and oblique to the osteon axis in both lamellar types, show parabolic distribution through specimen thickness. Longitudinal collagen in extinct lamellae, and transverse collagen in bright lamellae, peaks at middle third of lamellar thickness, while oblique collagen peaks at outer thirds of both types. Throughout the thickness, longitudinal collagen orientations characterize extinct lamellar specimens, while orientations oblique to the original osteon axis characterize bright lamellar specimens. Measured patterns complement previous indirect results by different methods and reinforce previously hypothesized differences in lamellar mechanical functions.

Keywords: Bone; Collagen; Confocal microscopy; Lamellae; Plywood structure; Polarized light; Secondary osteon

1. Introduction

Following Leeuwenhoek's early observations (1693), the lamellar structure of secondary osteons in human bone has remained the subject of numerous investigations. While researchers over the centuries have agreed on the existence of two lamellar types, different hypotheses as to the structural characteristics that differentiate one type from the other were presented. The debate focused principally on the organization of collagen fibrils and carbonated apatite crystallites, the main elementary components of secondary osteons.

The differences of opinion were sustained by the challenges: (1) to optimize microscopy technique and specimen preparation for structural visualization of lamellae whose thickness ranges between a mere 2 and 16 μm and whose shape is curved; (2) to interpret the microscopy observation of structure in terms of orientation and density of collagen and apatite; and (3) to extrapolate the 3-dimensional biological reality of macroscopic bone from the 2-dimensional information provided by the microscopy plane of focus on specimens excised from macroscopic bone at specific orientations and observed at specific angles.

To confront these challenges, investigators employed increasingly sophisticated microscopy tools with higher magnifications and resolutions as they became available. These tools have enabled acquisition of knowledge about the lamellar structure and provided the foundation for further refined structural hypotheses. Further, the

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development of materials science and engineering theories over time has allowed more robust testing and assessment of the hypotheses formulated by the various investigators. In recent decades, increasing computer capability and software sophistication have allowed simulation of mechanical testing of lamellae and osteons to include greater structural detail so as to explain function with higher accuracy.

This introduction reviews early compound microscopy investigations of lamellar structure, modern microscopy investigations, and previous lamellar modeling to provide context for the present research.

The earliest microscope of the 1590s was merely a tube with a plate for the object at one end, and at the other, a lens which yielded a magnification of less than ten times actual size. Leeuwenhoek devised techniques to grind and polish small lenses of great curvature that afforded his microscopes unprecedented 270× magnification. His examination of bone revealed osteons and their lamellae (Leeuwenhoek, 1693). Microscopes of the time used light to illuminate the bone specimen, and their magnifications were not bettered by more than an order of magnitude until the 20th century. The limitation on magnification was the wavelength of light. With white light, any two details closer than 0.275 μm are seen as a single detail by an ordinary light microscope, and any detail with a diameter smaller than 0.275 μm will be either invisible or blurry. Over time, the magnification available by compound microscopes became only approximately an order of magnitude higher, up to 1800× with ordinary light and up to 5000× with blue light.

Kölliker (1854), by regular light microscopy with magnifications up to 350×, perceived "lamellated" bone matrix with two layers in each lamella: one layer as pale and more homogeneous; a second layer as darker, granular and for the most part striated. He hypothesized that differences in elementary component densities might explain the difference in appearance. Ebner (1875) was one of the first researchers to employ polarization of light in a compound microscope for lamellar investigations. When light is forced to travel as a plane wave through a bone specimen placed between a so-called polarizer and a so-called analyzer, the image seen through the eye pieces of the microscope consists of either extinct or bright signals depending on whether the light passes through the specimen substructures. That the concentric lamellae of an osteon in cross-section gives rise to either an extinct or bright polarized light signal points to a difference in the structure of the underlying lamellae. However, the significance of the signal requires interpretation because the extinct or bright signal per se does not yield information about the structural differences that determine its appearance. By means of a magnification up to 600× under polarized light, Ebner interpreted the appearance of "lamellation" as due to orientation of "connective fibers." He hypothesized that the "connective fibers" were a component of bone tissue, separate from calcium salts that would lie in the spaces between the fibers.

Ranvier (1887) followed Kölliker's terminology of differentiation between homogeneous lamella and streaked lamella. At a magnification up to 600× under polarized light, he associated an extinct appearance to the streaked lamella and a bright appearance to the homogeneous lamella. He hypothesized that the different extinct or bright appearance is due to a difference of fiber orientation. Because fibers viewed transversely to their axis appear extinct, the homogenous lamella would appear extinct on a section transverse to the osteon axis by virtue of its fibers being viewed transversely to their axis. Because fibers viewed along their length appear bright, the streaked lamella would appear bright on a section transverse to the osteon axis by virtue of its fibers being viewed along their axis. This association of the polarization signal with fiber orientation was at that time a non-verified hypothesis derived through reasoning on the physics of the polarized light microscopy. Gebhardt (1906) also interpreted the differences in lamellar types, viewed through regular and polarized light microscopy, in terms of orientation of higher percentage components. More specifically he proposed what in modern terminology is defined as an orthogonal or quasi-orthogonal plywood osteonic model, where collagen fibrils change orientation from a lamella to the next one. Ziegler (1906) instead interpreted the different appearance of lamellae under polarized light as resulting from a difference in density associated with fibrillar lamellae separated by what he defined as interstitial substance, without fibrillae extending from a lamella to the next.

Weidenreich (1930) conducted his regular light microscopy investigations with up to 1040× magnification and supported Gebhardt's interpretation of difference as due to orientation of elementary components. Ruth (1947), through regular light microscopy of up to 1800× magnification differentiated between compact and diffuse lamellae in accordance with their structural appearance. He saw "compact lamellae" as bands of circumferentially oriented compact, felted or interwoven bundles of fibrillae, and "diffuse lamellae" as bands of radially oriented fibrillae, loosely disposed, and separated from each other by relatively wide interfibrillar spaces filled with a granular substance. The fibrillae themselves were observed as delicate strands disposed at right angles to the compact lamellae.

Amprino (1946) and Amprino and Engström (1952) addressed the issue of developing a technique to investigate carbonated apatite density. They developed a high-resolution micro-X-ray that showed variation of degree of calcification among secondary osteons. Engström and Engfeldt (1953) showed that X-ray absorption varies in lamellae. They hypothesized existence of lamellae with a high content of organic and inorganic material alternating with lamellae containing less substance.

The application in the 1950s of transmission electron microscopy to secondary osteons, some 20 years following its invention, offered a new level of insight. In this kind of microscope, electrons are accelerated in a vacuum until their wavelength is extremely short, only one