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Review

Open questions on the 3D structures of collagen containing vertebrate mineralized tissues: A perspective

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ABSTRACT

Our current understanding of the structures of vertebrate mineralized tissues is largely based on light microscopy/histology and projections of 3D structures onto 2D planes using electron microscopy. We know little about the fine details of these structures in 3D at the length scales of their basic building blocks, the inherent variations of structure within a tissue and the cell-extracellular tissue interfaces. This limits progress in understanding tissue formation, relating structure to mechanical and metabolic functions, and obtaining deeper insights into pathologies and the evolution of these tissues. In this perspective we identify and discuss a series of open questions pertaining to collagen containing vertebrate mineralized tissues that can be addressed using appropriate 3D structural determination methods. By so doing we hope to encourage more research into the 3D structures of mineralized vertebrate tissues.

1. Introduction

Vertebrate mineralized tissues are diverse and structurally complex. Their structures are hierarchically ordered, in some cases graded and the tissues are most often an intimate mix of cells, extracellular matrix and mineral. Furthermore, the extent of structural variation even within relatively small volumes, is high. Thus obtaining high resolution structural information on these tissues is challenging. However, such information is the basis for better understanding how the tissues form, how they function metabolically and mechanically, how they fail in pathology and how they evolved. The overall objective of this perspective is to identify open questions regarding the structure of collagen containing vertebrate mineralized tissues, that we believe can be resolved by using advanced methods of high resolution 3D structural studies. We hope that this perspective will stimulate more structural research on vertebrate mineralized tissues.

The four classes of mineralized vertebrate tissues, as defined by Hall (2005) are: bone, cartilage, dentin and enamel. Vertebrates also produce other mineralized tissues, including cementum which covers the root dentin of teeth, inner ear otoliths and otoconia, and eggshells in birds. The foundation of our structural understanding of these tissues was developed in the late 19th and early 20th Centuries and still dominates our current concepts regarding their structural diversity, development and evolutionary changes. Much of what we know about these tissues is based on plain and polarized light microscope analyses of thin sections (histology); methods that have been available in some cases for 150 years e.g. (Le Gros Clark, 1945; Schmidt, 1924). The introduction of soft X-ray imaging, X-ray diffraction, and scanning and transmission electron microscopy, certainly added much more detail, but for the most part has not radically changed the basic concepts. Both light and electron imaging methods are still based mainly on 2D observations or projections of 3D structures onto a 2D plane. Due to the complex, graded and hierarchical structure of many vertebrate mineralized tissues, 2D observations are sometimes hard to interpret. The bone family of materials is probably the best studied among all the vertebrate mineralized tissues. The structures of some of these bone materials are among the first to be studied in detail in 3 dimensions (3D) and therefore provide a perspective on what we may discover when other vertebrate mineralized tissues are examined in 3D.

2. Methodological options for high resolution 3D structural determinations of vertebrate mineralized tissues: A brief analysis

In order to understand the structures of many vertebrate mineralized materials, we need to be able to resolve the details of the basic building block – the mineralized collagen fibril. In bone, these fibrils are around a 100 nm in diameter and they have a repeat structure around 67 nm (Hodge and Petruska, 1963; Orgel et al., 2006). We also need information on the crystals – their shapes and preferred orientations. The crystals are around tens of nanometers long and wide and

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only 3–4nm thick (Fratzl et al., 1992; Weiner and Price, 1986). We need also to be able to study cells, non-mineralized extracellular matrices and the interphases between the cells and the matrices. Finally, we need to know the spatial relationships between the collagen fibrils, mineral platelets and cells within the bone matrix. Furthermore, these studies should provide detailed 3D information and include as large a volume of tissue as possible. Fulfilling all these requirements with one analytical technique is not possible. Here we briefly present our analysis of the more promising methodological options that address as many of the above requirements as possible. For a detailed systematic review and analysis of many of the methodological options available for 3D structural analysis of bone, see Georgiadis et al. (2017).

High resolution confocal light microscopy can be used to image cells and the extracellular matrix in 3D with sub-micron resolution. It is also possible to image fluorescently labelled specific macromolecules using confocal microscopy, as well as supra-resolution light microscopy, thus extending this approach to the nanometer resolution level for the labelled entities. Confocal microscopy can also be combined with Raman spectroscopy to obtain localized chemical information from labelled entities (Bennet et al., 2014), and polarized Raman spectroscopy can provide information on collagen orientation (Masic et al., 2011). A particularly interesting option is created when incorporating a fluorescence detector in the Raman microscope so that the volumes around fluorescently labelled molecules or cell entities can be chemically analysed (Bennet et al., 2014). Preferred orientation can be determined using second harmonic generation (SHG) imaging. This is of particular interest for characterizing collagen orientation in bone and other tissues (Zoumi et al., 2002).

MicroCT is optimal for 3D imaging relatively large volumes of highly contrasting materials such as mineralized tissues to a resolution of around a micron. Some instruments can achieve higher resolution, but for much smaller volumes and have also been applied to the study of bone (Schneider et al., 2007). By introducing a phase contrast component, some of the more prominent elements of soft tissues (such as the fibers or sheets), that have not been fixed or stained, can also be imaged in 3D at the 1 μm resolution range – a major advantage for the study of mineralized tissues (Naveh et al., 2014).

High resolution images are usually produced using an electron beam and the obtainable resolution is certainly sufficient to image the collagen fibrils, the crystals and the details of intracellular components. The main techniques using an electron beam to produce 3D data are tilt tomography in the transmission and scanning transmission electron microscopes (TEM and STEM), and serial sectioning and imaging using adaptations of the scanning electron microscope (SEM). By chemical fixing or cryo-fixing of the tissue to be studied, both cells and their components, as well as the extracellular matrix and mineralized matrix can be imaged. These capabilities are certainly of direct relevance to understanding the 3D structures of mineralized tissues.

To date, one of the most promising approaches to obtain high resolution 3D structural information of mineralized vertebrate tissues using an electron beam is serial sectioning of an embedded tissue. This can be achieved using a microtome inside an SEM (serial block face SEM, SBF SEM) (Denk and Horstmann, 2004), or by serially sectioning the tissue using an ion beam that removes a thin layer followed by imaging using an SEM (FIB SEM) (Heymann et al., 2006; Stokes et al., 2006). For both methods, the images are aligned in a stack, which can then be interrogated in 3D. The thickness of the sections in SBF SEM is between 25 and 200 nm (Hughes et al., 2014), and in FIB SEM the thickness can reach 3 nm (Zeiss Crossbeam FIB SEM technical data). This determines the resolution in the Z direction. The resolution in the X and Y directions is set by the resolution of the SEM and can also be around 3–5 nm. Sample preparation usually involves fixing, staining for contrast, high pressure freezing and freeze substitution. The volumes that can be practically studied by all these methods vary from around ten microns cubed at the highest resolution to several tens of microns cubed. Currently, the major limitations of both techniques (SBF SEM and FIB SEM) regarding vertebrate mineralized tissues are that the mineral needs to be removed prior to embedding in order to visualize the matrix, and the matrix can only be visualized if a heavy metal contrasting agent is introduced uniformly into the volume under study. The latter is a non-trivial process. The major advantages are that high resolution structural information of both cells and matrices in relatively large volumes can be obtained.

Imaging vitrified tissue volumes without the need for fixing and staining is ideal for mineralized tissues, both because of the need to maintain the water content and not induce mineral precipitation or dissolution artifacts, and to also preserve as well as possible the cells and extracellular matrices. Cryo-FIB SEM is now possible (Schertel et al., 2013). We predict that cryo FIB SEM will contribute enormously to our understanding of vertebrate tissues. The first studies are encouraging (Vidavsky et al., 2016). The cryo-fixation option also resolves the challenging requirement of uniformly staining dense tissues through large volumes. Cryo-FIB SEM however cannot, as yet, resolve the matrix structure without prior removal of the mineral. For higher resolution, but only for volumes whose thicknesses are around 300 nm or less, cryo-thin sections can be studied in the TEM using tomography (Mahamid et al., 2015). The cryo-FIB SEM can be used to produce location-specific thin sections by cutting and section thinning using the heavy atom beam.

An alternative option for obtaining 3D information is to use X-ray beams as high-resolution probes to indirectly infer mainly orientational structural information in 3D volumes. This requires a very narrow X-ray beam and a tomographic approach. There are several such options based on SAXS and WAXS (Georgiadis et al., 2017), but the output is mainly the predominant orientations of the crystals and the macromolecules at any given location. Predominant orientations are however insufficient to characterize a complex structure, such as for example lamellar bone, where the different orientations of all the fibrous components are an integral part of the structure. Furthermore, poorly oriented components in association with oriented components, as well as different characteristic textures, could easily be overlooked. The ability to use a rapidly scanning X-ray beam to obtain many SAXS and WAXS patterns over a 2D surface, does however provide much insight into the graded nature of many vertebrate mineralized tissues (Pabisch et al., 2016; Tesch et al., 2001). There is also the exciting possibility of 3D SAXS mapping of a sequence of surfaces (Fratzl et al., 1996; Georgiadis et al., 2015; Schaff et al., 2015). See also comments on this method (Fratzl, 2015).

A technique which may well be applicable to vertebrate mineralized tissue structures is cryo- soft X-ray tomography (cryo- SXT) (Pereiro and Chichón, 2014). This technique can provide 3D tomographic information on cryo-preserved samples at a spatial resolution of 30 nm. The thickness of the sample can be up to 30 μm. A major advantage is that in addition to the structural information, chemical information can also be obtained on the 3D distribution of certain elements such as calcium (but not phosphorus), as calcium is not affected by the strong absorption of water (Pereiro and Chichón, 2014).

A most promising approach for comprehensively studying vertebrate mineralized tissues is to combine different methods in a correlative manner, namely image the exact same volumes in 3D using two or more imaging methods. These correlative methods can involve imaging fluorescently labelled molecules with confocal microscopy and then locating the label at high resolution in 3D using FIB SEM (Weiner et al., 2016). Another option is to combine light microscopy and micro-CT (Pierantoni et al., 2016). It should also be possible to carry out elemental analyses using EDS in a correlative manner with cryo-FIB SEM and obtain chemical information on the solutions within and between cells; an exciting future prospect for the study of vertebrate mineralized tissues.