Temporal analysis of bone development in chick femur bone using label-free imaging

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Background incl. aims:
Long bones such as femur develop via the process of endochondral ossification (EO). During EO, chondrocytes proliferate, undergo hypertrophy and subsequent cell death with the replacement of hyaline cartilage with bone. The constructed cartilaginous matrix is invaded by blood vessels, osteoblasts, osteoclasts, and bone marrow cells before it develops into bone. Currently, the gold standard techniques to study organ development are micro computed tomography, histochemical assays, or through fluorescence microscopy using dyes or endogenously expressed fluorescent proteins protein. However, all these approaches are invasive, and can require fixation or lysis of tissue, therefore are unsuitable for in-vivo studies on humans for monitoring disease progression or therapeutic benefit.

However, multiphoton microscopy utilising non-ionising, benign radiation (NIR light) offers non-invasive, label-free structural and chemical details, without damage in contrast to histological approaches and X-ray scans. Additionally, label-free microscopy facilitates high sensitivity and resolution. In this work, we report an application of multiphoton microscopy with the techniques of Second Harmonic Generation (SHG), Coherent Anti-Stokes Raman Scattering (CARS) and 2-Photon Excited Auto-Fluorescence (2PaF) to monitor bone development. We establish the methodology using the chick model to provide proof-of-concept of its capability for potential use with patients.

Methods:
The femurs of chick embryos were isolated at different growth phases, i.e., at day 11, 14, and 18. The bones were fixed, embedded in paraffin wax, and sectioned. The sectioned slices were imaged with histological methods to establish the ground truth. The parallel sections were imaged with SHG, CARS and 2PaF. SHG images collagen fibres while CARS is used to image distribution of lipids at their vibrational frequency of 2845cm-1 and 2PaF images autofluorescence at 520 nm (primarily FAD and NADH). CARS was obtained in both backward and forward scattering geometries. Subsequently, label-free imaging data was analysed using image analysis using Fiji software. SHG images were analysed using CT-FIRE software and statistical analysis were performed.
Results:
Histological staining (Alcian Blue and Sirius Red) revealed a clear demarcation between the early cartilaginous femur and the surrounding tissue. Proteoglycans were visible within the cartilage region and intense collagen staining in the tissue surrounding the femur.

SHG images of chick femur bones (n=6) at Day 11, 14 and 18 highlighting collagen fibres were analysed using “CT-FIRE” to extract collagen fibre details. It was observed that the length of collagen fibres steadily increased throughout the developmental process from Day 11 to Day 18, unlike the fibre width that remained similar for early developmental stages before sudden increase at day 18. However, there were no observed differences in fibre straightness throughout the developmental process. 2PaF images provided context for cells and tissue areas while CARS images showed the distribution of lipids and protein structures and provided a readout of changes on development from Day 11 to 18. Further analysis is in progress to develop imaging based multimodal signatures for bone development.

Conclusions:
Our work shows that non-invasive and non-destructive, structurally, and chemically selective multiphoton label-free imaging techniques can be advantageous for monitoring bone formation and can track its development. Additionally, the label-free techniques explored here allow objective and quantitative analysis of the developing bone since signals are retrieved only from molecules and structures in their native state providing intrinsic contrast and are dependent on their amounts.

Graphic:

Keywords:

Bone development, Multiphoton-microscopy, label-free imaging,

Reference:

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