14th Biennial Conference on High Resolution X-ray Diffraction and Imaging
XTOP 2018
3rd – 7th September 2018
Bari, Italy

X-RAY COMPUTED TOMOGRAPHY FOR QUANTITATIVE ANALYSIS OF 3D CELLS DISTRIBUTION IN CARTILAGE FOR STUDYING DEVELOPMENT IN VERTEBRATES

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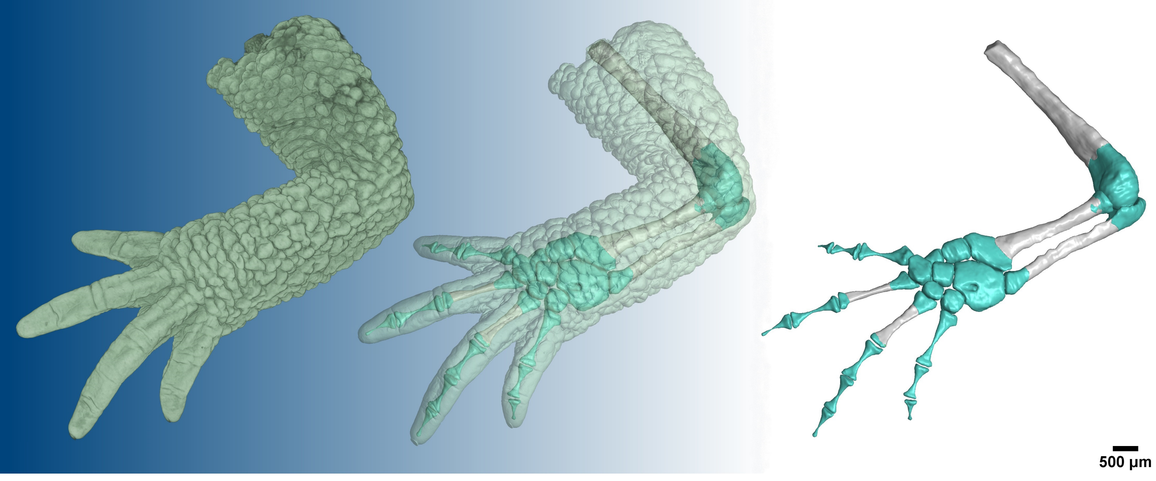
Abstract

One of the goal of modern developmental biology is to reveal how the geometry of skeletal structures is controlled during growth and regeneration. Finding a way how to visualize, quantify and analyze these structures with cellular resolution in three-dimensions (3D) might shed a new light to explaining theories focusing on developmental and comparative biology. In this work, synchrotron X-ray computed microtomography (µCT) and chemical contrasting has been exploited for a quantitative analysis of the 3D-cell distribution in tissues of a developing salamander (*Pleurodeles waltl*) limb – a key model organism for vertebrate regeneration studies. We mapped the limb muscles, their size and shape as well as the number and density of cells within the extracellular matrix of the developing cartilage. By using tomographic approach, we explored the polarity of the cells in 3D, in relation to the structure of developing joints. We found that the polarity of chondrocytes correlates with the planes in joint surfaces and also changes along the length of the cartilaginous elements. Our approach generates data for the precise computer simulations of muscle-skeletal regeneration using cell dynamics models, which is necessary for the understanding how anisotropic growth results in the precise shapes of skeletal structures.



I Introduction

A number of improvements in last years have developed X-ray-based methods for cellular imaging. X-rays can penetrate cells and thick tissues (from millimeter- to centimeter-sized samples) without the need for sectioning the sample. X-ray computed microtomography (µCT) is a non-destructive imaging method that provides high spatial resolution (from micron to sub-micron scale) of 3D data for samples with the wide size ranging (basic principle of this method is illustrated in Figure 1). Recent developments of this method have significantly advanced biological imaging.

Here, we took advantage of the synchrotron-based X-ray µCT technique in combination with chemical contrasting in mapping the cells, their orientation and extracellular matrix distribution in 3D, during salamander limb development in the simultaneous analysis of cartilage and muscles. The method allowed for the mapping and 3D-reconstruction of several different tissue types at the same type, which is essential for understanding the development of a muscle-skeletal system.

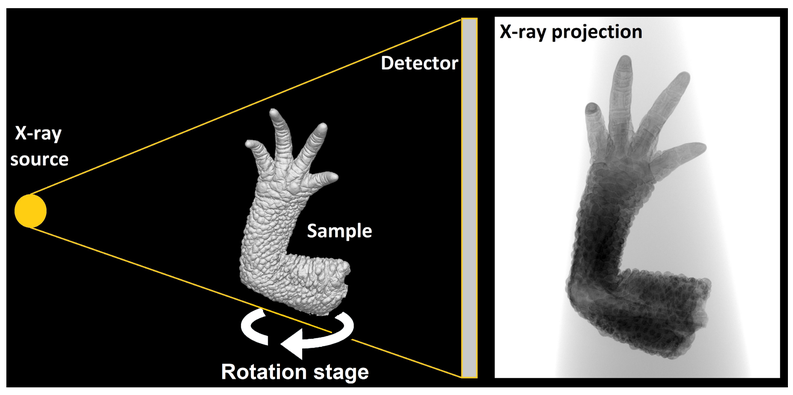


Fig. 1. Schematic of set-up for conventional X-ray imaging
The beam generated by X-ray source creates a projection on detector. The sample rotates on rotation stage

II Conventional and synchrotron microtomography

Because of the easier access to an industrial rather than a synchrotron CT device, a conventional device was used for the first experiments with salamander limbs. First, the quality of staining was checked on an X-ray projection (Figure 2). After that, multiple trials were realized on µCT GE v|tome|x L 240 and the exceptional quality of chemical contrasting of the samples was achieved. In this case the resolution provided by a µCT turned out to be insufficient to analyse the shape development and growth when taking into account the cellular and matrix proportion per locality. Still, the resolution delivered by µCT was near-cellular, which meant that with a synchrotron beam it would most likely achieve the cellular resolution for the necessary analysis.

The high photon flux, X-ray beam geometry and high spatial resolution (down to 1 µm) of synchrotron µCT at SYRMEP beamline of Elettra achieved the cellular resolution for a quantitative analysis of cell distribution. Comparison of conventional and synchrotron data is in Figure 3.

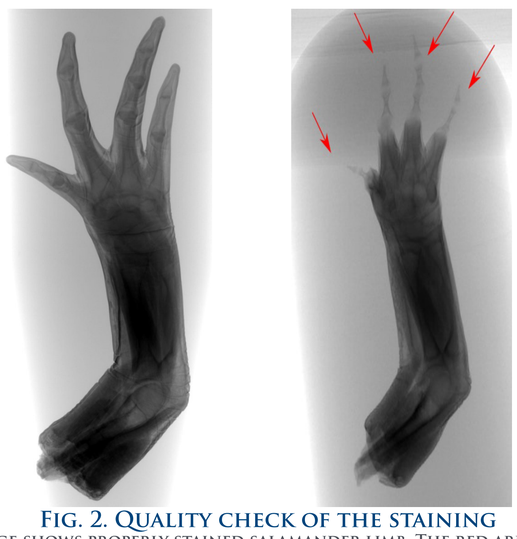


Fig. 2. Quality check of the staining
The left image shows properly stained salamander limb. The red arrows on right image show unstained tips of the fingers..

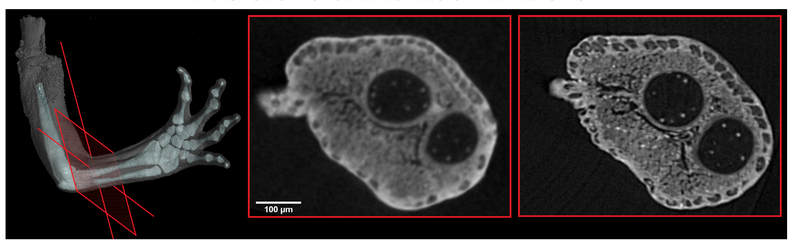


Fig. 3. Comparison of conventional and synchrotron data
Tomographic slice of the salamander limb: left image shows slice obtained by conventional X-ray machine. Right image shows data from synchrotron.

III Data analysis

Obtaining high-contrast tomographic data is the first step for desired 3D analysis. Another no less important part is data processing of reconstructed tomographic slices. To perform quantification of the data, the analysis can be divide into four main steps:

- Segmentation of cartilaginous elements - AB Snake.
- Segmentation of cells inside these elements - Pore3D.
- Quantification of the segmented cells - Pore3D.
- 3D visualization - VG Studio.

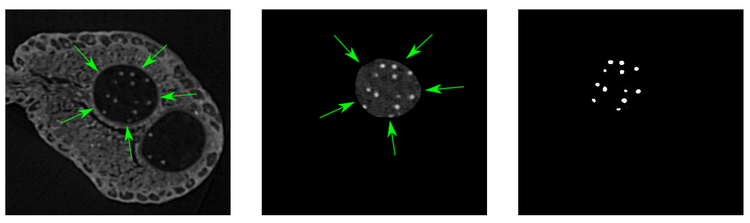


Fig.4. Individual segmentation steps
A tomographic slice (left), One cartilage element (middle) and segmented cell nuclei (Right). The green arrows show one element to be segmented.

IV Results

In this study, we have demonstrated a novel, technical approach allowing for the quantitative analysis of 3D cell distribution inside the whole developing muscle-cartilaginous units from a regenerative animal model. This approach is based on the chemical contrasting of samples with phosphotungstic acid (PTA), followed by a high-resolution X-ray microtomography (µCT) measurement and the subsequent 3D data-processing and analysis.

Quantification of the developing cartilage elements includes:

- Number of cells
- Density
- Polarization
- Zonal distribution
- Total volume of cartilaginous elements
- Mapping of muscle attachment points

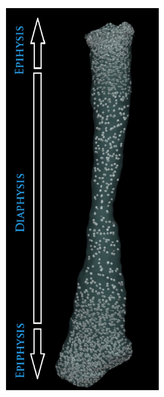


Table 1. Results of quantitative analysis.

	Epiphysis	Diaphysis	Epihysis
Number of cells	888	429	595
Total volume [mm^3]	0.0052	0.0071	0.0027
Average size of one cell [mm^3]	$5.81x10^{-6}$	$1.65x10^{-6}$	$4.47x10^{-6}$
Density of cells [mm^3]	171,296	60,791	233,684

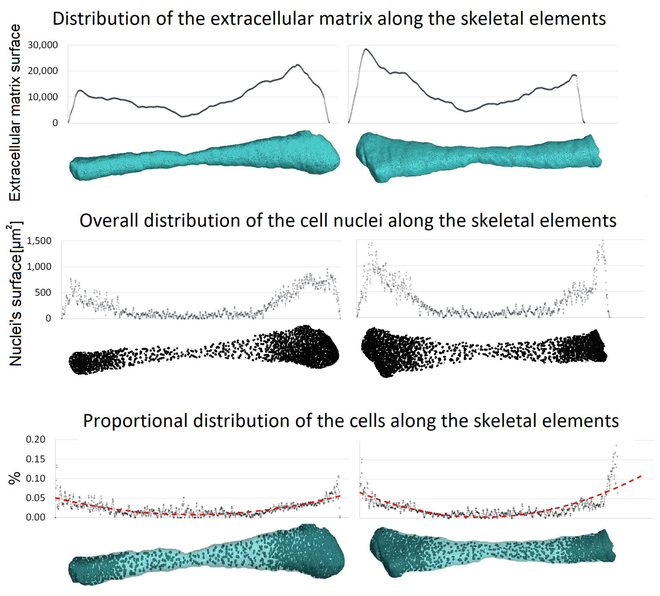


Fig. 5. Distribution of extracellular matrix and cell nuclei along skeletal elements
Top: distribution of the area of extracellular matrix. Middle: distribution of number of cells represented by the area of cell nuclei. Bottom: ratio between the area of cell nuclei and the area of extracellular matrix.

V Muscle-skeletal connection

Our visualizations provided detailed information about developing joint surfaces at cellular resolution in 3D in the salamander limb. Development of surface geometry correlated in time with the formation of attached striated muscles. Orientation of chondrocytes in the developing joint correlated with the changing curvatures of joint surfaces. More generally, the resolution and differential contrast were sufficient to map the orientation of all chondrocytes within the cartilage, which provided important foundation for future inference of the oriented cell behaviour during cartilage shaping. Our results demonstrated that the predominant orientation of chondrocytes in epiphyseal regions was different from rather central regions of the cartilage, where the cell density appeared low. In addition to this, superficial chondrocytes in epiphyseal regions were aligned with the developing surface of the cartilage (Figure 6 and 7).

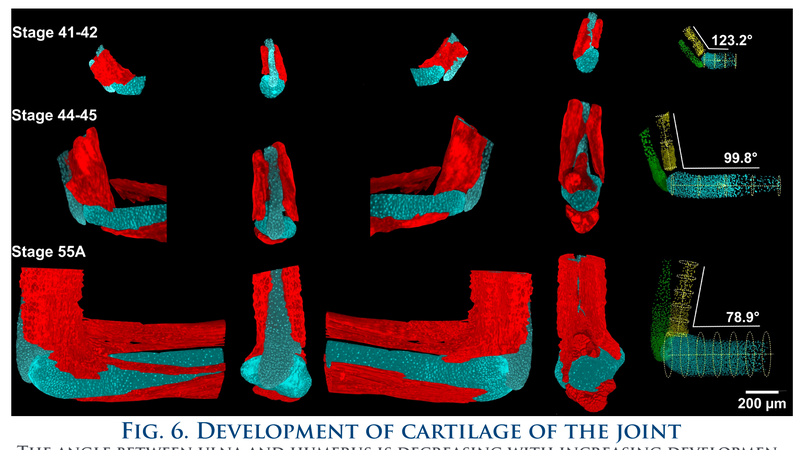


Fig. 6. Development of cartilage of the joint
The angle between ulna and humerus is decreasing with increasing developmental stage. There is a correlation between decreasing angle and splitting of the muscles.

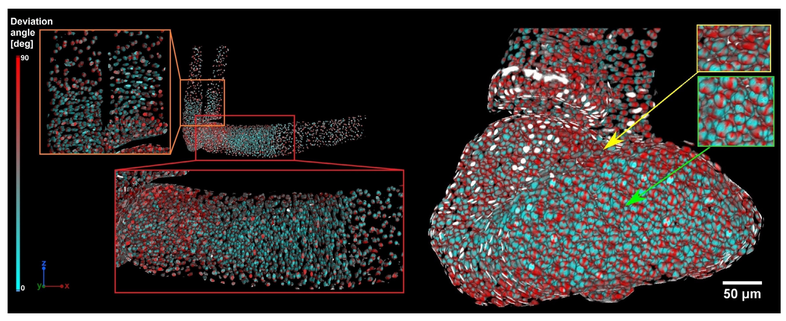


Fig. 7. Polarization of the cells inside cartilage
There are polarized zones between diaphysis and epiphysis. Detail views on orientation of the cells shows that superficial chondrocytes near surface (yellow arrow) are aligned with the developing surface of the cartilage in the contrary of chondrocytes in the middle of the cartilage (green arrow).

Big advantage of microtomography measurement enhanced by staining is a large variety of tissues that can be detected. The information about all structures in the sample is included in one dataset obtained by one single measurement. This allows to show the position and the arrangement of the structures inside the sample. We show organization of cartilage with fibers of muscles surrounded by one-cell layer of skin epithelium inside the arm of salamander's embryonic limb. Such a 3D model, which is composed by sub-models gives new perspective how to look inside the biological structures.

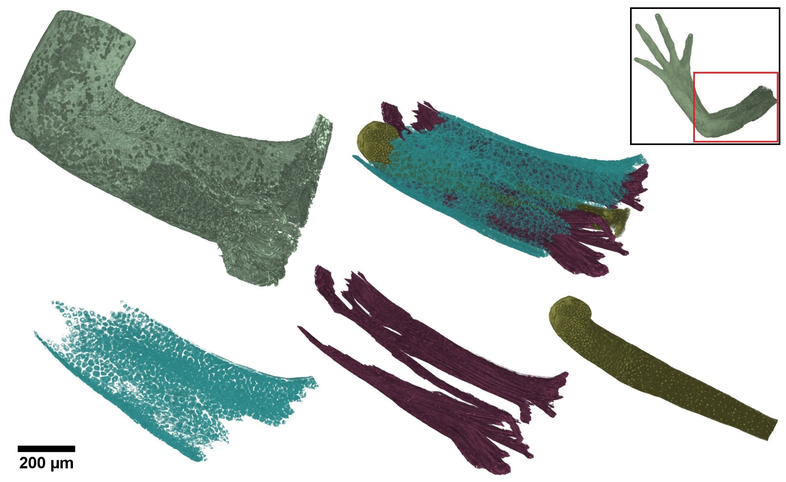


Fig. 8. variety of tissues that can be visualized in 3D
Cartilage (yellow) , fibers of muscles (pink), one-cell layer of skin epithelium (blue) of limb of embryonic salamander.

References:

1.Tesařová M. *et al.*, A quantitative analysis of 3D-cell distribution in regenerating muscle-skeletal system with synchrotron X-ray computed microtomography, *Sci. Rep*. (2018) *under review*.

Acknowledgment

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II and CEITEC Nano Research Infrastructure (MEYS CR, 2016-2019).

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