Application note

A novel approach for the 3D visualization of the developing skeleton

For years, clearing and staining with Alizarin red and Alcian blue have been the standard method for the imaging of vertebrate and whole-embryo skeletal development.[1,2] Staining allows images with enormous anatomic detail to be obtained, which can then be used to characterize animal models and developmental malformations. However, most of this work has been confined to 2D imaging, limiting the amount of information that can be obtained.

Novel methods capable of visualizing bone and cartilage in 3D present a groundbreaking opportunity to enhance our understanding of the processes underlying vertebrate development.[1,2] The transition from 2D to 3D imaging offers researchers a whole new dimension of information, and would open exciting avenues in developmental biology, comparative anatomy, and in the identification of novel markers in developmental disorders.

Refining the process

Making use of optical projection tomography, several recent studies have effectively produced 3D images of Alcian-blue stained cartilage in cleared specimens.[3] Other approaches have explored the use of varying modalities such as micro-computed tomography (microCT), 3D phase-contrast X-ray tomography, and magnetic resonance (MRI). MicroCT can also be used for the 3D imaging of developing bones, allowing genuine 3D data to be collected at micrometer resolution.

When it comes to dependable separation and intensity-based visualization of skeletal tissues, there has not been a standardized workflow that enabled simultaneous, isotropic 3D imaging of cartilage and bone at similar quality.[4]

For this reason, a research team led by Stephan Handschuh at the University of Veterinary Medicine has developed a streamlined X-ray microCT imaging protocol for the 3D characterization of skeletal tissues.4 In this approach, the cartilage matrix is selectively labeled in order to provide it with sufficient contrast over other tissues, enabling automated visualization and segmentation while sparing the bone mineral. This allows both tissues in the developing skeleton to be imaged simultaneously.

To establish this protocol, various fixatives, washing agents, and staining solutions were tested. The optimized staining protocol was found to consist of ruthenium red in 50% ethanol, applied to ethanol-fixed samples.[4] The use of this protocol was demonstrated on E16.5 mouse fetuses; the team utilized Thermo Scientific™ Amira™ Software for 3D reconstruction and analysis.

Drawing distinctions

With this approach, deep anatomic details of the skeleton could be visualized, including regions critical to the study of skeletal development, such as the limbs and ribcage. Morphometric measurements of these skeletal elements were then planned, but overlap between fetal bone and cartilage in the X-ray attenuation made this challenging. A dual-energy protocol was therefore used to separate bone from cartilage, taking advantage of the varying X-ray attenuation properties of ruthenium and hydroxyapatite at the two energies. The extracted material fractions offered well-separated ruthenium and hydroxyapatite signals, which were then used to generate 3D renderings of the samples. These closely resemble the results obtained with clearing and staining, but have the added benefit of authentic, isotropic 3D data that can be used for accurate morphometric measurements. Overall, the protocol was able to separate the cartilage matrix from most organs and soft tissues.

This is the first instance of a high-contrast staining protocol capable of both intensity-based segmentation and visual phenotyping of cartilage elements, enabling the quantitative and qualitative analysis of cartilage development.[1]

A promising alternative

Due to the simple and reproducible nature of this staining protocol, this method could be used for fully automated imaging and quantitative morphometric analysis, as well as other high-throughput analyses.[1] Additionally, while the protocol could feasibly be use with lab-based scanners already, further hardware developments could improve image quality.

The information that can be obtained from genuine 3D data is incredibly valuable for the study of skeletal development, and could find application in areas such as screening for developmental disorders and the quantitative characterization of development malformations. For example, this approach would allow for the detection of abnormalities in the limbs and ribcage, which tend to be studied in genetic models of disease or as a part of developmental toxicity studies.

Clearly, this novel workflow is a promising effective alternative to more conventional phenotyping of vertebrate skeletal development, proving invaluable insights for various life science fields.

Thermo Fisher Technology

Amira Software empowered the Handschuh team with a powerful platform for 2D–5D image visualization, analysis, and processing across a wide variety of imaging modalities. A range of built-in options, including segmentation and surface-area-volume tools, provided critical 3D data processing capabilities.

Delivering flexibility and speed, Amira Software supports advanced imaging workflows for a number of research areas, including structural and cellular biology, tissue imaging, bioengineering, and preclinical imaging. Its powerful segmentation and image processing capabilities and workflows allow you to gain vital insights into your data.

References

1. Hilton, M. J. (2014). *Skeletal Development and Repair.* https://doi.org/10.1007/978-1-62703-989-5
2. Yamazaki, Y., *et al.* (2011). *Whole-mount bone and cartilage staining of chick embryos with minimal decalcification,* **Biotechnic and Histochemistry**, 86(5), 351–358. https://doi.org/10.3109/10520295.2010.506158
3. Akhter, M. P., & Recker, R. R. (2021). *High resolution imaging in bone tissue research-review.* Bone, 143(August 2020), 115620. https://doi.org/10.1016/j.bone.2020.115620
4. Gabner S, *et al. The visible skeleton 2.0: phenotyping of cartilage and bone in fixed vertebrate embryos and foetuses based on X-ray microCT,.* **Development** 2020;147:dev187633. doi:10.1242/dev.187633.

Learn more at **thermofisher.com/amira**

thermo scientific