Whole Joint 3D Neurovascular Mapping in Musculoskeletal Systems

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INTRODUCTION: Musculoskeletal diseases are the leading cause of disability around the world. Pain, altered tissue structure, and compromised joint functions are the major symptoms suffering patients with musculoskeletal diseases. Despite decades of research efforts in the musculoskeletal field, the underlying disease mechanism remains elusive, partly due to the complexity of the musculoskeletal system and the lack of integrated approaches to investigate the problem at the whole joint level. Neurovascular networks play critical roles in joint health and disease development. Nerves are essential to joint homeostasis and pain sensation, while vascularization serves the needed oxygen, nutrients, hormones, and other biomolecules for cell/tissue maintenance and contributes to joint degeneration as well. Given spatial heterogeneity at the joint and tissue levels, a comprehensive study of the 3D neurovascular network is critical to investigate the tissue or region-specific mechanisms and build an integrated mechanistic understanding of the musculoskeletal system. However, there is very limited knowledge of neurovascular networks from a 3D whole joint perspective. Current studies usually utilize the traditional histological approach to investigate the distribution of nerves and blood vessels in tissue sections. However, such a method is 2D based and the sectioning process is invasive, which provides only limited information on a section of the joint tissues and leads to sample distortion. 3D imaging of musculoskeletal tissues using advanced microscope systems is promising, but the imaging depth is restricted to hundreds of micrometers due to the tissue light scattering issues. Although information from a series of tissue sections can be combined to reconstruct a 3D view of the sample, this strategy is time-consuming, and the invasiveness issue remains unresolved. More importantly, none of those methods can provide the whole joint 3D structure and morphology information and, therefore, fail to interpret the interplay among different tissue types. Along with the development of light sheet microscopy and tissue clearing techniques, noninvasive 3D imaging of neurovascular networks at a whole joint level is emerging. Several studies have investigated 3D innervation but are limited to single joint tissue components, such as the femur bone. 3D neurovascular mapping at a whole joint level is still challenging as the different joint tissue components require different strategies for clearing and imaging. In this study, we hypothesize that 3D neurovascular mapping at a whole joint level is achievable with large-field light sheet imaging and tissue-clearing approaches. We successfully developed a tissue clearing and imaging approach for 3D joint imaging and mapped the 3D nerves and blood vessels in multiple musculoskeletal systems, including the knee joint, temporomandibular joint (TMJ), and sacrum. Both overview and fine nerve and blood vessel structures were observed and registered with the corresponding joint tissue component.

METHODS: Fresh rat knees, TMJs, and sacrum samples were harvested from Sprague Dawley rats (2 months old, ~ 210g) at the conclusion of other IACUC-approved research projects at the Medical University of South Carolina. Samples were then immediately processed and fixed in a 10% neutral buffer formalin solution. Following decalcification, deep permeabilization, decolorization, and delipidation, the whole joint samples were immunostained with antibodies for beta-3 tubulin (TUBB3) and calcitonin gene-related peptide (CGRP). Then, samples were cleared and imaged with a large-field light sheet microscope. Images were processed and rendered using Imaris (Bitplane, UK) and ImageJ (NIH) software.

RESULTS: Rat knee joint, TMJ, and sacrum samples were immunostained with antibodies for nerve fibers and successfully cleared and imaged with a large-field light sheet microscope. 3D imaging results of whole knee samples showed the fine nerve fiber networks in the periosteum and the outer layer and attachment tissues of the meniscus while no nerve fibers were seen in the inner layer of the meniscus (**Fig. 1**). We also found blood vessel-like networks using the autofluorescence signals imaged with the 488 nm laser excitation (**Fig. 1C** and **1D**). Overlapping both nerve and vessel signals revealed the interactions between nerves and blood vessels: nerves run along and surround the vessels (**Fig. 1D**, vessels highlighted with white arrows). Results with TMJ samples also demonstrated the innervation of CGRP+ nerves in the anterior and posterior bands of the TMJ disc (**Fig. 2**). The capsule tissues in both lateral and medial sides of TMJ also showed the presence of nerve fibers. The retrodiscal tissues have high nerve density. The condyle bone was further segmented from the images and innervation was co-registered with joint morphology (**Fig. 2C**). The imaging of sacrum samples was used as a demonstration of the application of the method in spine-related tissues (**Fig. 3**). We can clearly see the symbolical innervation pattern in the sacrum with two large nerve bundles symmetrically running along the axial length of the sacrum. CGRP+ and Tubulin+ nerves innervate the space between two sacrum bone sections (**Fig. 3C**).

DISCUSSION: In this study, we have developed a 3D method to stain, clear, and image the neurovascular networks in musculoskeletal systems at the whole joint level in rats. Compared to 2D approaches, this 3D imaging approach is unbiased and noninvasive, therefore allowing a more accurate description of the complex network structure, more precise quantification of the interested features, and much less chance of missing some rare events or phenomena. Our results elaborated on the complexity of the neurovascular networks in musculoskeletal systems. The innervation and vasculature pattern varies among different tissue components and is site-specific or heterogeneous. Ongoing studies are using immunolabeling techniques to enhance the contrast of blood vessels and will include the investigation of other nerve types, such as sympathetic nerves. This method is also scalable, being able to be applied to large joint specimens from large animal models and humans. Studies on the injury or disease effects on the 3D whole joint neurovascular structure are in the process.

SIGNIFICANCE/CLINICAL RELEVANCE: The 3D mapping of the neurovascular network in the musculoskeletal system will advance our understanding of tissue-specific functions and their contributions to the whole joint health and disease development and assist the development of new treatment strategies for musculoskeletal diseases.
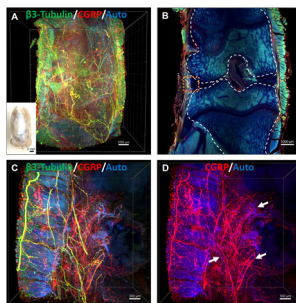
Fig. 1 **A** 3D view of neurovascular networks in the rat knee (inserted image). **B** Single frame of the 3D images shown in **A**. The dashed line shows the bone contour. **C** and **D** 3D high-resolution images of neurovascular structures in the meniscus regions (dashed square in **B**).
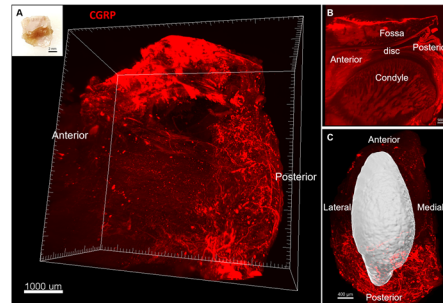
Fig. 2 **A** 3D view of neurovascular networks in the rat TMJ (inserted image). **B** Single frame of the 3D images shown in **A**. **C** 3D top-view of neurovascular structures of TMJ disc and surrounding tissues. The condyle was shown as the white solid.
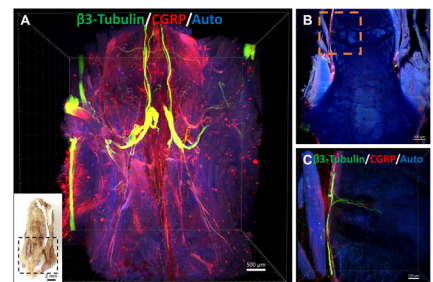
Fig. 3 **A** 3D view of neurovascular networks in the rat sacrum (inserted image). **B** Single frame of the 3D images shown in **A**. **C** 3D images of nerves innervate the space between two sacrum bone sections (dashed square shown in **B**).