A Multimodal Toolbox for Mapping and Profiling Murine Joint Innervation

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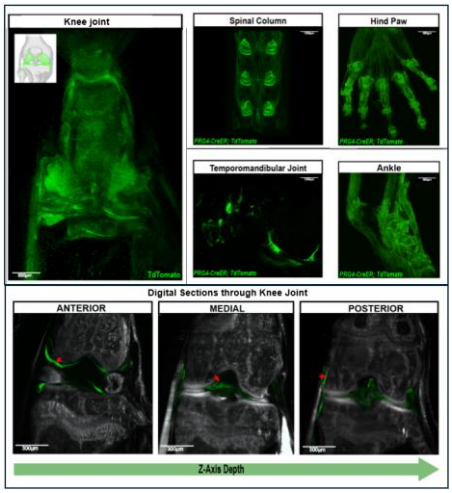
INTRODUCTION: Joint pain is an increasing concern for our aging population, as current therapies to slow joint disease progression or reduce pain are largely ineffective and often carry significant health and dependency risks. Age and joint disease induce changes to all tissues that make up the joint, including the dense neural network that innevates the joint. Several studies have correlated some joint innervation changes in joint diseases such as osteoarthritis or rheumatoid arthritis, but little is known about how they affect the individual's pain experience. In addition, these studies typically focused on a single neural subtype due to a limited availability of validated tools to study joint innervation. To better understand the relationship between aging, joint disease, and pain, systematic characterization of pain nociceptors and other neural subtypes that regulate joint homeostasis and pain signaling is urgently needed. This study's goal was to establish a validated methodological toolbox for accurate mapping of the neuro-architecture in the murine knee.

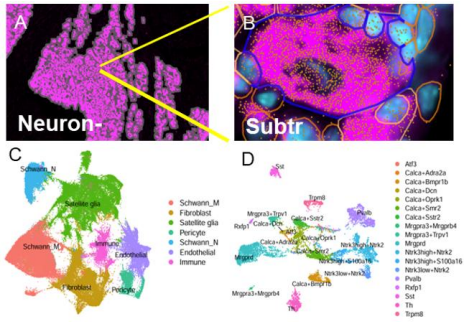
METHODS: We took a multimodal approach to establish a validated joint innervation toolbox. First, we screened through molecular and genetic tools to identify and/or label various sensory neuronal subtypes in the mouse knee joint. We screened a panel of genetic reporter mice for peripheral nociceptors or post-ganglionic sympathetic neurons, assessing their specificity and accuracy for labeling their respective neural subtype in the dorsal root ganglion (DRG) and knee joint. We also compared the performance of a series of conventional retrograde tracers for effective tracing of primary afferent sensory and post-ganglionic sympathetic neurons from the knee joint. In parallel, we developed a novel method for efficient clearing and 3D-imaging of complex musculoskeletal tissues. Starting from EZ Clear, a simple and rapid aqueous-based tissue clearing method for soft tissues, we performed iterative method modifications to define optimal conditions for fixation, decalcification, and delipidation of intact mouse hindlimbs. Lastly, we developed methods for multi-omic profiling of joint-innervating sensory neurons and the cells they interact with in the DRG. To do so, we adopted existing snRNAseq protocols and developed novel methods for spatial transcriptomic analysis of the mouse DRG.
All experiments were performed on a minimum of 3 animals per parameter for 1 biological sex (typically male). All animal experiments were validated in the other sex. All procedures involving animal subjects were carried out according to protocols approved by Baylor College of Medicine IACUC committee.



KneEZ Clear efficiently clears intact mouse joints.

RESULTS: We created a multimodal joint innervation toolbox that includes:

1. A validated set of genetic and molecular resources to label sensory and autonomic neuronal subtypes that innervate the mouse joint. We screened 6 distinct mouse genetic reporter lines, used in the neuroscience field to label distinct sensory and autonomic neuronal subtypes, as well as 5 conventional retrotrade tracers, and assessed their efficiency and specificity for labeling knee joint-innervating neurons in the mouse knee and dorsal root ganglion. Our study revealed that Fast Blue and True Blue are the most reliable and efficient retrograde tracers, while the Scn10a^{tm2Cre} mouse line shows the highest specificity for labeling nociceptive neurons in the mouse knee and DRG. Importantly, our data revealed extensive non-specificity for many reporter lines, requiring co-labeling with a pan-neuronal marker to reliably label knee joint-innervating neurons.
2. A simple, flexible tissue clearing method for whole joints. KneEZ Clear is highly flexible and efficiently clears multiple heterogeneous musculoskeletal tissues, such as the mouse knee joint, vertebral column, hindlimb, skull, and teeth. This method does not require specialized equipment and retains endogenous signal from fluorophores and fluorescent proteins.
3. Reliable multi-omic methods for profiling mouse DRGs. To enable in-depth, multi-omic characterization of joint-associated tissues, we optimized single nucleus RNAsequencing (snRNAseq) and spatial single cell transcriptomics of the mouse DRG. Based on our snRNAseq analyses, we developed an optimized gene panel (~400 genes) for spatial transcriptomic analysis of the mouse DRG. To aid future spatial studies, we are currently in the process of generating the first high-resolution, single-cell spatial map of the mouse DRG.



Spatial transcriptomic profiling using our custom gene panel identifies all known cell types in the DRG.

DISCUSSION: This study highlights the numerous challenges related to mapping joint innervation and provides solutions to overcome many of these obstacles. The data presented here showcases validated, efficient tools for labeling several neuronal subtypes of neurons. However, we show significant limitations of existing genetic reporter lines. Hence, additional efforts will be needed to create novel, reliable genetic reporter lines to label additional neuronal subtypes. Our joint innervation toolbox also includes methods for whole joint tissue clearing and imaging, as well as for multi-omic profiling of the sensory neuronal cell bodies in the DRG. Together, these tools will enable reliable, multimodal characterization of mouse knee joint-innervating neurons from the mouse knee joint to the DRG.

SIGNIFICANCE/CLINICAL RELEVANCE: The validated methods, tools, and resources identified in this study will facilitate the creation of comprehensive joint innervation maps in physiological and pathological contexts, setting the stage for identifying the cellular and molecular changes responsible for mediating joint pain.

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