Optimization of nuclei isolation from human skeletal muscle, infrapatellar fat pad, and synovium for multi-omics methods

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INTRODUCTION: Mapping cellular heterogeneity and molecular profiling is essential for understanding the etiology and pathogenesis of musculoskeletal disease. Single-nucleus (sn) sequencing offers a strategy to interrogate transcriptional and epigenetic profiles in frozen clinical specimens where intact cell isolation is challenging. For example, when cells are too large to be encapsulated (*e.g.*, muscle fibers), or when adipocytes are a key feature of the tissue, such as in the infrapatellar fat pad in osteoarthritis, or intramuscular fatty infiltration in rotator cuff tears or chronic low back pain. However, sn-RNA or sn-ATAC sequencing require robust nuclei isolation protocols to ensure unbiased and sufficient yield and quality. Unfortunately, many studies do not report nuclei yields per mg of tissue, and if they do, yields appear lower than what would be expected based on nuclei counts from histology. Low yields are a major bottleneck to understanding musculoskeletal disease because the transcriptional and epigenetic data derived from them may not be representative of the nuclei in the tissue. Therefore, the goal of this project was to optimize a nuclei isolation protocol and report absolute yields per mg of tissue, so that future studies can benchmark their yields, or even better, improve them by further optimizing the isolation protocol.

METHODS: Human skeletal muscle, synovium, and infrapatellar fat pad samples were obtained under IRB-approved protocols with informed consent. A total of 12 donors were included (6 male, 6 female). Tissues were collected intraoperatively during ACL reconstructions (healthy semitendinosus, fat pad, and synovium), vertebral fusion (degenerated multifidus) and total knee arthroplasty (fat pad and synovium). Approximately 15–50 mg of frozen tissue was processed using optimized nuclei isolation protocols tailored for each tissue type. The nuclei isolation kit by 10x Genomics and 50 mg of tissue were utilized as a starting point. Nuclei concentration and cell viability (as a marker for insufficiently lysed tissue) were assessed using a fluorescence cell counter. Yields are reported in absolute terms as nuclei per mg of starting tissue and as a concentration per uL which is critical for 10x protocols where carry-forward volumes are limited. The optimization process was started with skeletal muscle, then applied to fat pad, and finally, synovium.

RESULTS SECTION: Across all samples and tissue types, optimized protocols produced nuclei suspensions suitable for downstream genomics workflows. In healthy skeletal muscle, we were able to isolate >11,000 nuclei per mg, in degenerated multifidus muscle, >4,000 nuclei/mg were obtained. Yields for fat pad were >10,000 nuclei/mg and >6,000 nuclei for synovium, respectively (Fig. 1). The most critical modifications to the standard protocol were decreasing lysis time, adding liberase to the lysis buffer, and utilizing specialized BioMasher pestles which facilitate the homogenization process. For the 10x Genomics workflow, the carry-forward volume is limited to only 5 uL, therefore, nuclei concentration had to be maximized which was challenging because resuspension efficiency decreased when smaller volumes were used. However, reducing resuspension volume from 50 uL to >10 uL increased concentration by 3-fold (data not shown). Input mass was gradually reduced and high yields from as little as 15 mg of tissue were achieved (Fig. 1B).

DISCUSSION: High nuclei yields are critical and numbers per mg of tissue should be reported in publications. Low nuclei yields indicate that the isolation was not aggressive enough so that only the nuclei "easiest-to-release" were obtained, or conversely, that they represent the last surviving nuclei after too aggressive isolation. Thus, sn-RNA or sn-ATAC data from small yields may not represent the true nuclei profiles within the tissue. The protocol optimizations performed may not be exhaustive, but they serve as a starting point for future isolations and optimization, and currently reported yields were exceeded by 3-5-fold. Furthermore, variability across tissue types underscores the need for tissue-specific optimization. It was particularly challenging to obtain nuclei from more fibrous/fatty skeletal muscle tissue which were more mechanically resistant to pestling.

SIGNIFICANCE/CLINICAL RELEVANCE: As little as 15 mg of tissue was sufficient to obtain similar yields compared with 30-50 mg of tissue. This mass corresponds to a small biopsy as it can be taken clinically for diagnostic purposes (or for research).

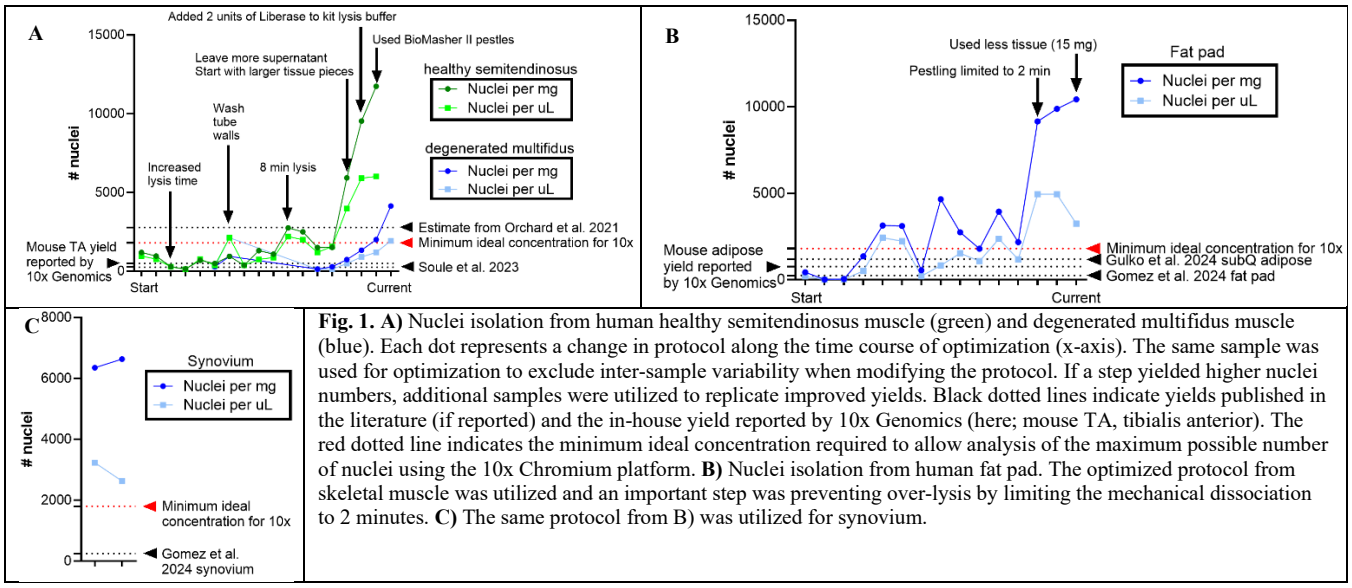

Fig. 1. A) Nuclei isolation from human healthy semitendinosus muscle (green) and degenerated multifidus muscle (blue). Each dot represents a change in protocol along the time course of optimization (x-axis). The same sample was used for optimization to exclude inter-sample variability when modifying the protocol. If a step yielded higher nuclei numbers, additional samples were utilized to replicate improved yields. Black dotted lines indicate yields published in the literature (if reported) and the in-house yield reported by 10x Genomics (here; mouse TA, tibialis anterior). The red dotted line indicates the minimum ideal concentration required to allow analysis of the maximum possible number of nuclei using the 10x Chromium platform. **B)** Nuclei isolation from human fat pad. The optimized protocol from skeletal muscle was utilized and an important step was preventing over-lysis by limiting the mechanical dissociation to 2 minutes. **C)** The same protocol from B) was utilized for synovium.