Towards Standardizing Cartilage Histomorphometry in Mouse Knees

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INTRODUCTION: Cartilage histomorphometry is a computer-based method to quantify cartilage degeneration and regeneration at the tissue and cellular level[1,2]. With the emerging technologies of digital pathology and machine learning, standardizing cartilage histomorphometry is becoming increasingly critical to compare results across studies and appropriately develop machine learning models for automation. Standardization of the cartilage histomorphometry pipeline still needs to be developed and validated, particularly in the preclinical setting. Previous research has exploited cartilage histomorphometry techniques in mouse knees and rat elbows to unlock key spatial, cellular, structural, and compositional aspects of cartilage degeneration[1,2]. However, cartilage histomorphometry must be more scalable for high-throughput analysis and integration with machine learning. In addition, cartilage histomorphometry methods need to be developed in open-source software and programming languages to enable widespread adoption by researchers. Thus, this study aimed to develop a high-throughput pipeline for cartilage histomorphometry in mouse knees using open-source software (i.e., QuPath[3]) and programming language (i.e., Python).

METHODS: *Joints and histological processing:* Knee joints were harvested from mice (n = 12; 16 wks of age) that underwent the destabilizing of the medial meniscus (DMM) to induce osteoarthritis (8 wks post-DMM) as part of our ongoing studies (IACUC approved); joints from contralateral limbs served as controls. Joints were processed for paraffin-embedding, sectioned (midsagittal at 5 µm) through the medial compartment, and stained with Safranin-O/Fast Green to identify cartilage and bone. *Histomorphometry:* Stained sections (n = 3/joint; 100 µm apart) were digitized using an Axio Slide scanner at 20x magnification (0.22 µm/pixel) and imported into the open-source software of QuPath to visualize and trace cartilage tissues. A standardized region of interest (500 µm wide and parallel to the tibia growth plate) was located at the mid-contact region of the tibia and femur (Fig. 1A). Articular cartilage and calcified cartilage of the femur and tibia were manually traced. Smaller boxed regions were placed in the underlying subchondral bone and the synovial joint space to serve as a background to normalize the image color space. *Data analysis and statistics:* Images and traced cartilage and background masks were exported from QuPath and imported into Python programming language to perform the following sequentially (Fig. 1B): i) rotate images/masks such that the tibia calcified cartilage is on the bottom of the image and approximately parallel to the x-axis; ii) scale the image intensities to the joint space and bone; iii) threshold the scaled image to isolate proteoglycan-rich areas (i.e., positive safranin-o staining); and iv) calculate cartilage area and proteoglycan-rich area. Furthermore, isolation of the interfaces between each tissue and joint space (i.e., articular surface, tidemark, and subchondral bone; Fig. 1C) enabled the evaluation of the interface's jaggedness by fitting each interface line to a polynomial line. Once fit, the standard deviation of each interface line's pixel distance from the fitted line was quantified, whereby increased standard deviation indicates increased interface jaggedness. Unpaired t-tests were performed to detect differences in each histomorphometry (averaged across sections) between injured and control cartilage. Statistical significance was set at $p < 0.05$.

RESULTS: Qualitative observations confirmed articular cartilage surface irregularities and proteoglycan loss in both the femur and tibia typically associated with DMM-induced osteoarthritis (Fig. 2A). Histomorphometry of cartilage revealed a significant reduction in total cartilage and proteoglycan-rich areas of the articular cartilage in both femur and tibia (Fig. 2B); no significant changes were observed in the calcified cartilage (data not shown). Quantification of interface jaggedness highlighted that in the femur, DMM-injured joints had an increase and decrease in the jaggedness of the cartilage/bone interface and tidemark, respectively, with no change in the articular surface (Fig. 2B); in the tibia, only the cartilage/bone interface was increased (Fig. 2B).

DISCUSSION: This study demonstrated the standardization of cartilage histomorphometry in mouse knees by quantifying structure (area and tissue interface jaggedness) and composition (proteoglycan area) using open-source software and programming. Quantitative metrics confirmed localized changes in the tibia articular cartilage structure and composition previously seen from the coronal plane in DMM-injured mouse joints[1]. The current study identified a similar, if not worse, cartilage damage in the femur not quantified previously[1]. While no overt structure and composition changes were seen in the calcified cartilage of both the femur and tibia, the alterations in the jaggedness of the interfaces stemming from the calcified cartilage (i.e., the tidemark and subchondral bone interface) suggest tissue structural changes not captured previously. Ongoing work aims to apply this study's histomorphometry method across a broader range of DMM-induced osteoarthritis studies in our group and evaluate the spatial component of such changes (i.e., anterior vs. posterior and medial vs. lateral). Furthermore, we are using the traced cartilages to develop machine learning models to segment cartilage automatically.

SIGNIFICANCE/CLINICAL RELEVANCE: This study lays the foundation for standardizing cartilage histomorphometry in the mouse knee, helping to develop machine learning models for evaluating cartilage unbiasedly. Automating cartilage histomorphometry preclinically will accelerate the development of novel strategies for treating and reversing osteoarthritis clinically.

REFERENCES: [1] David+, *JOR,* 2016; [2] David+, *Front. Bioeng. Biotechnol,* 2022**;** [3] Bankhead+, *Sci.Rep.,* 2016

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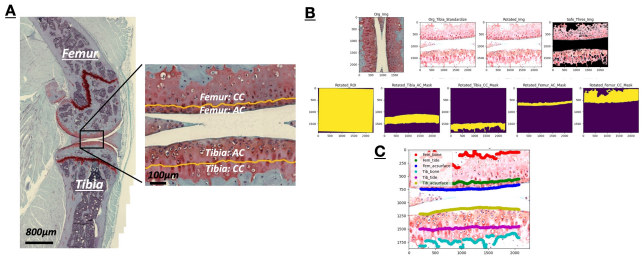

Fig. 1: Generalized Pipeline for Cartilage Histomorphometry. (A) Represented image of a mouse knee joint showing the region of interest for analysis (black box) and the cartilages traced (zoomed image); cc = calcified cartilage, ac = articular cartilage. (B) Example of image rotation and color scaling to standardize the tibia/calcified cartilage orientation with the x-axis of image and to threshold the image for positive Safranin-O staining. (C) Example image highlighting the interface lines used to calculate the interface jaggedness of cartilage/bone interface (red/turquoise), tidemark (green/purple), and cartilage surface (blue/gold).

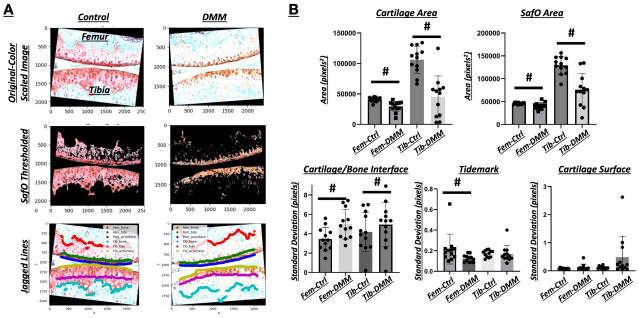

Fig. 2: Histomorphometry of DMM-induced Cartilage Changes. (A) Represented images of control and DMM highlighting the scaled image, safranin-O (SafO) thresholded image, and jaggedness of cartilage/bone interface (red/turquoise), tidemark (green/purple), and cartilage surface (blue/gold); extra black pixels in the images (i.e., padded image) are due to image processing during rotation and ignored in the analysis. (B) Quantitative metrics of cartilage area, SafO positive area, and interface jaggedness. # = $p < 0.05$ compared to control.