Methodology: Collagen Fibril Orientation Analysis

Image Processing and Quantitative Analysis of PLM Images

Collagen fibril orientation was quantified from Polarized Light Microscopy (PLM) images through a systematic image processing pipeline based on HSV (Hue, Saturation, Value) color space analysis. Each PLM image was first converted from BGR to HSV color space using OpenCV, where the Hue channel (0–179° in OpenCV convention) encodes the birefringence-dependent color information directly correlated with collagen fibril orientation. To eliminate background noise and non-birefringent regions, a dual-threshold mask was applied, excluding pixels with saturation ($S < 10$) or value ($V < 10$). The fibril orientation angle (θ, ranging from 0° to 90°) was calculated through linear interpolation of the hue values, where the hue corresponding to the Superficial Zone (SZ) was mapped to 0° (fibrils parallel to the articular surface) and the hue corresponding to the Deep Zone (DZ) was mapped to 90° (fibrils perpendicular to the tidemark), following the equation:

$$\theta = [(H - H_0) / (H_{90} - H_0)] \times 90° \qquad (1)$$

where H is the measured hue value, H_0 is the reference hue for SZ (0° orientation), and H_{90} is the reference hue for DZ (90° orientation). To ensure robust hue averaging across the circular color space, circular mean calculations were employed using trigonometric functions to prevent artifacts at hue boundaries. The cartilage depth was normalized to tissue thickness and discretized into 100 equally spaced bins, with each bin analyzed independently to generate continuous depth-dependent orientation profiles. For each depth bin, the mean orientation angle ($\bar{\theta}$), standard deviation (σ), and representative color properties (RGB values, hex color, and intensity) were computed from all valid pixels within the corresponding horizontal slice.

The articular cartilage was segmented into three anatomically distinct zones based on normalized tissue thickness: the **Superficial Zone** (SZ, 0–10%), the **Middle Zone** (MZ, 10–40%), and the **Deep Zone** (DZ, 40–100%). To establish baseline orientation profiles for comparative analysis, one representative PLM image was selected as a reference for each healthy cartilage group—specifically, healthy Femoral Cartilage and healthy Tibial Cartilage. These reference images underwent the complete orientation analysis pipeline to establish the characteristic depth-dependent fibril angle profiles (θ vs. normalized depth) for healthy tissue. Subsequently, the fibril orientation profiles of degraded

cartilage specimens, categorized by disease progression as **Early Osteoarthritis (EOA)**, **Moderate Osteoarthritis (MOA)**, and **Advanced Osteoarthritis (AOA)**, were analyzed using identical processing parameters (zone boundaries and color calibration) derived from their respective healthy references. For both femoral and tibial compartments, the degraded group profiles were directly overlaid and statistically compared against the healthy reference profile via depth-wise mean angle calculations and standard deviation propagation. This reference-based comparative approach enabled quantitative assessment of zone-specific alterations in collagen fibril architecture—including disorganization, loss of surface tangential orientation, and disruption of the characteristic arcade-like structure—as a function of osteoarthritis severity progression.