# Spatial Distribution of Newly Synthesized Glycosaminoglycans in Cartilage

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**INTRODUCTION**: Proteoglycans in articular cartilage are essential for the weight-bearing and lubrication functions of tissue [1,2]. The content of glycosaminoglycans (GAGs), the major type of proteoglycans in cartilage, is an important indicator of cartilage and neo-cartilage qualities [3]. Current immunological and biochemical methods have limitations on studying the spatial distribution of newly synthesized GAGs in cartilage. We previously developed a click chemistry-based technique to label the newly synthesized GAGs by incorporating fluorescence into GAG chains. In this study, to evaluate the spatial distribution of new GAGs and its alteration during osteoarthritis progression, we developed an imaging technique to visualize and quantify the labeled GAGs. The imaging technique was also used to measure the changes in chondrocyte cell volume and nuclear volume after inflammatory challenge.

**METHODS**: *Sample preparation*: Cylindrical cartilage samples (diameter = 3 mm, thickness = 2 mm, middle zone) were harvested from the femoral condyles of calf (1 to 2 months old) knee joints and incubated in chondrogenic medium [4]. IL-1β (10 ng/mL), a pro-inflammatory cytokine, was supplied in the medium to simulate joint inflammation. *Fluorescent labeling of new GAGs*: Samples (n = 3/group) were first cultured in medium containing an azide-modified monosaccharide (GalNAz), a building block for the GAG disaccharide units. During the 24-hour culture, GalNAz was taken up by chondrocytes and incorporated into the newly synthesized chondroitin sulfate in GAGs. A fluorescent dye, AZ488, was "clicked" to the azide groups on the new GAG chains (Fig. 1a) [5]. Chondrocytes cell body and nucleus (n = 6 explants/group) were labeled with CellTracker™ (Invitrogen) and Hoechst (Invitrogen), respectively. *Confocal imaging and image processing:* Z stacks of fluorescently labeled cartilage samples were captured on a confocal microscope (Zeiss LSM 880) every 0.52 µm with ~150 slices. A custom-designed, NIH Fiji-based image processing protocol was developed to measure the volumes of new GAGs, chondrocytes, and nucleus. First, various filters were applied to reduce image noise, enhance contrast, and remove artifacts. An automated local threshold technique was applied to segment the z stacks into object interiors and exteriors. Cell clusters were detached, and separated cell components were re-connected based on the Distance Transform Watershed algorithm. A 3D ellipsoid fitting method was applied to analyze chondrocyte cell volume and nuclear volume. *Quantification of GAG content*: To quantify the newly synthesized GAG content, click chemistry labeled cartilage was digested, and the fluorescent intensity of the digestion medium was read with a plate reader. To track the longitudinal loss of GAGs from cartilage under inflammatory challenge, the click chemistry-labeled tissue was cultured in IL-1β medium, and the fluorescent intensity of culture medium was read every other day.

**RESULTS**: The newly synthesized GAGs encircled cells in cartilage matrix (Fig. 2a). After the tissue was treated by IL-1β for 2 or 7 days, the green halo, representing the newly synthesized GAGs, shrank compared to the control (Fig. 2a). Image processing showed that 2- and 7-day IL-1β treated samples had significantly smaller new GAG volume (46% and 41% of control, respectively) (Fig. 2b), despite having brighter new GAG halos than the control (Fig. 2a). According to the fluorescent reading from the plate reader, the new GAG contents in the 2- and 7-day treated samples were significantly reduced to 88% and 76% of the control, respectively (Fig. 2c). Another batch of samples were first labeled for new GAGs and then treated with IL-1β (Fig. 2d). Two- and 7-day IL-1β treatment significantly reduced the GAG volume to 61% and 70% of the untreated control, respectively (Fig. 2e). According to the fluorescent reading of culture medium, loss of GAG contents after 2- and 7-day treatments are 50% and 160% higher than the control, respectively (Fig. 2f). Chondrocyte cell volume decreased after 2- or 7-day IL-1β treatment (Fig. 3). Nuclear volume also decreased after 7-day IL-1β treatment, but not after 2-day treatment (Fig. 3). Chondrocyte and nuclear volumes in the control group, without IL-1β treatment, showed no significant changes after 7-day *in vitro* tissue culture.

**DISCUSSION**: This study developed a new fluorescent labeling technique and NIH Fiji-based image processing protocol to visualize the spatial distribution of newly synthesized GAGs in articular cartilage. Newly synthesized GAGs encircled chondrocytes and were more concentrated under inflammatory challenge (Fig 2a,b,d,e). GalNAz was mainly incorporated into chondroitin sulfate. In cartilage, 90% of chondroitin sulfate is present in the aggrecans [6]. Thus, the spatial distribution of labeled GAGs in the image stacks represents the distribution of newly synthesized aggrecans. Our results showed that IL-1β challenge can influence aggrecan synthesis and extracellular assembly by reducing the spatial volume and increasing the density of new aggrecans in areas next to plasma membrane (Fig. 2a,b,d,e). IL-1β treated cartilage showed GAG volume reduction and content loss in the first 2-day treatment (Fig. 2e,f). With longer 7-day IL-1β challenge, GAG volume shrinkage ceased while the loss of GAG content continued, indicated by the reduced halo brightness and the increased fluorescence in culture medium (Fig. 2d,e,f). IL-1β treatment also reduced the chondrocyte and nuclear volumes (Fig. 3), which could be related to *in situ* chondrocyte apoptosis under inflammatory challenge [7].

**SIGNIFICANCE/CLINICAL RELEVANCE**: This study developed a new GAG-labeling technique and NIH Fiji-based image processing protocol to quantify the spatial distribution of newly synthesized GAGs in cartilage, representing a new tool to study the metabolic behaviors of cells.

**REFERENCES**: [1] Kiani+ 2002. [2] Roughley+ 1994. [3] Ramaswamy+ 2008. [4] Zhou+ 2015. [5] Porter+ 2022. [6] Heinegard + 2009. [7] Yu+ 2000.

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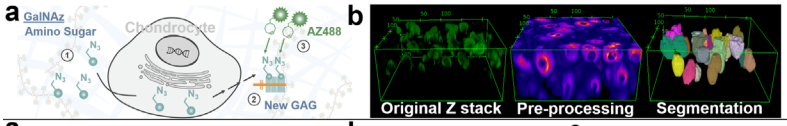


**Fig. 1 (a)** Illustration of GAGs labeling with click chemistry method. Modified amino sugar, GalNAz, is taken up by chondrocytes and incorporated into new GAG chains, that are then labeled with fluorescent dye AZ488. **(b)** Image processing and segmentation of newly synthesized GAG.

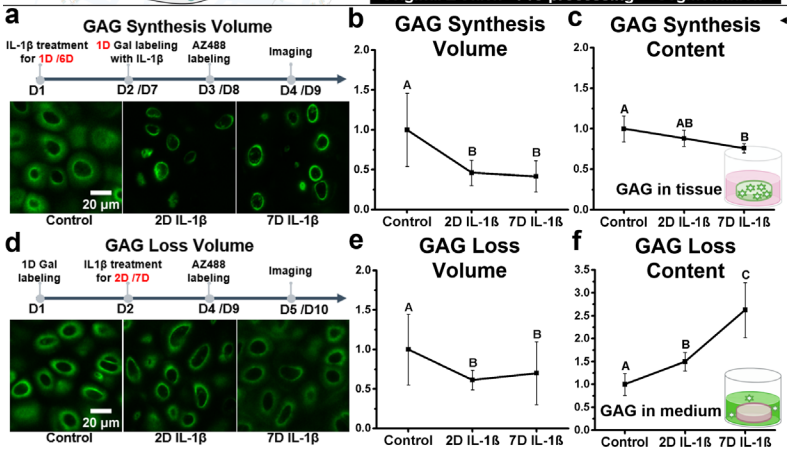


**Fig. 2 (a)** Newly synthesized GAGs (green) in cartilage after 2- and 7-day IL-1β treatment. **(b)**Volume of new GAG measured with confocal image stacks. **(c)** GAG synthesis rate quantified by fluorescent plate reader. **(d)** Labeled GAGs in cartilage after 2- and 7-day IL-1β treatment. **(e)** Volume of labeled GAGs. **(f)** Loss of GAG content measured by fluorescent reading of culture medium. (mean ± SD, complete GAG objects ≥ 32 from 3 cartilage explants).

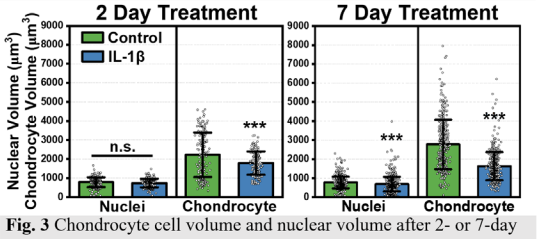


**Fig. 3** Chondrocyte cell volume and nuclear volume after 2- or 7-day IL-1β treatment. (mean ± SD, total number of chondrocytes ≥ 32 from 3 cartilage explants, ***: $p < 0.001$, n.s.: not significant).