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Nonlinear optical microscopy of early stage (ICRS Grade-I) osteoarthritic human cartilage

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Abstract: In a synovial joint, the articular cartilage is directly affected during the progression of Osteoarthritis (OA). The characterization of early stage modification in extra-cellular matrix of cartilage is essential for detection as well as understanding the progression of disease. The objective of this study is to demonstrate the potential and capability of nonlinear optical microscopy for the morphological investigation of early stage osteoarthritic cartilage. ICRS Grade-I cartilage sections were obtained from the femoral condyle of the human knee. The surface of articular cartilage was imaged by second harmonic generation and two-photon excited fluorescence microscopy. Novel morphological features like microsplits and wrinkles were observed, which would otherwise not be visible in other clinical imaging modalities (e.g., CT, MRI, ultrasound and arthroscope. The presence of superficial layer with distinct collagen fibrils parallel to the articular surface in 4 specimens out of 14 specimens, indicates that different phases of OA within ICRS Grade-I can be detected by SHG microscopy. All together, the observed novel morphologies in early stage osteoarthritic cartilage indicates that SHG microscopy might be a significant tool for the assessment of cartilage disorder.

OCIS codes: (000.1900) Diagnostic applications of nonlinear optics; (000.4580) Optical diagnostics for medicine; (000.4315) Nonlinear microscopy.

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1. Introduction

Osteoarthritis (OA) is a complex musculoskeletal disorder whose origin is not clear. It is one of the fastest growing major health conditions and a cause of huge socioeconomic burden [1].

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It is one of the most common rheumatic diseases that directly involve the articular cartilage [2]. Articular cartilage is typically divided into three zones: superficial, middle and deep. In the superficial zone, near the articular surface, the collagen fibers are mostly aligned parallel to the surface and contribute in resisting shear stresses. In the middle zone, the fibers are more randomly aligned to resist isotropic pressure from compressive loads, and in the deep zone the fibers aligned perpendicular to the surface plays an important role in securing the cartilage to the bone by anchoring the collagen fibrils to the subchondral bone [3, 4]. Articular cartilage covers the end of the long bones and act as a smooth (low friction) bearing surface to facilitate the movement of the joint. Macroscopically, fibrillation, erosion and progressive thinning of articular cartilage are known features associated with progression of OA [5, 6].

Although the gross morphological changes are known, relatively little is known about the underlying mechanism associated with the progression of OA. The inherent heterogeneity and slow evolution of the joint disorder amplified by lack of accurate characterization methods enhance the complications involved in early diagnosis. In fact, during the early stage of the disease, there is no "gold standard" available which can provide a clear dichotomy between those with and without the disorder [7]. In current clinical practice, a combination of clinical symptoms (pain, stiffening, swelling, crepitus, deformities etc.) and test (e.g., blood/urine) are being used for the diagnosis of OA and rule out other forms of arthritis.

While radiography remains a useful and inexpensive method for the diagnosis of OA, it is not useful for early diagnosis due to lack of sensitivity. Magnetic resonance imaging (MRI) shows considerable promise but limited resolution makes it incapable of reproducible measurements of early changes in articular cartilage [8].Therefore, development of a new method or technology which can allow more sensitive cartilage assessment to evaluate the disease progression and associated clinical feature is highly desirable.

Due to the noncentrosymmetric structure and ordered organization, collagen fibers are able to generate strong second harmonic signals [9]. The physical origin and mathematical treatment of second harmonic generation (SHG) in biological tissues is described elsewhere [9, 10]. Since, collagen is one of the major components of cartilage (~60% of dry weight), SHG microscopy can provide vital information about the structure and morphology of the extracellular matrix (ECM) of articular cartilage. Furthermore, the inherent confocality associated with nonlinear optical phenomena makes SHG as well as two-photon excited fluorescence (TPEF) microscopy capable of optical sectioning and thus generating three dimensional (3D) high resolution (~1 μm) optical images. Moreover, because SHG and TPEF signals originate from the endogenous molecules present in the cartilage matrix, no external label or dye is required, making this approach a label-free optical technique. These features (i.e., high resolution, high specificity and sensitivity, label free, 3D-optical sectioning capability) indicate that nonlinear optical microscopy (NLOM) is a candidate for the assessment of cartilage disorders including OA.

Earlier, a few studies have used NLOM to characterize cartilage. Yeh et al., demonstrated alteration in ECM of advanced degenerative bovine articular cartilage [11]. Jessica et al., demonstrated morphological changes in degenerative equine articular cartilage [12]. Brown et al., has investigated collagen meshwork architecture by a two-dimensional spring–mass network model in bovine high grade osteoarthritic cartilage [13]. Kiyomatsu et al., presented a quantitative approach to characterize induced OA in a mice model [14]. Additionally, a few other reports use NLOM to study degenerative cartilage, but the analysis is rudimentary [15–17]. Due to different load-bearing tendency in a synovial joint and the slow progression of OA, the pathophysiology in humans may differ from animal models and remains to be explored [18, 19]. Therefore, the investigation of human articular cartilage rather than an animal model, especially with early stage of osteoarthritic cartilage, is essential and potentially more relevant for clinical applications.

The development of NLOM probe based on multiphoton excitation that uses a near infrared laser beam for imaging is in progress by several groups [20–22]. An important factor

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that limits the use of NLOM probe in clinical studies is the lack of a compactness and flexibility. Jung et al., reported a miniaturized multiphoton microscopy probe that was developed by employing a microelectromechanical system scanning mirror and a double-clad photonic crystal fiber [20]. The size of probe head was 1 cm in outer diameter and 14 cm in length [20]. As another development, a miniaturized probe that possesses a diameter of 0.4 mm was developed by Bao et al., for two-photon-excited fluorescence imaging [22]. Such miniaturized and flexible probe may be advantageous for future *in vivo* NLOM based arthroscopy for the assessment of cartilage disorder.

In a previous study, we have shown the potential of polarization-SHG microscopy for the detection of fibrocartilage in early stage of OA [23] In the present study, we have performed a pilot experimental investigation of early stage (ICRS Grade-I) osteoarthritic articular cartilage obtained from the human knee. The aim of the investigation is to explore the capabilities of NLOM to characterize morphological and structural changes during early stage of OA.

2. Materials and methods

The use of human samples was approved by the Regional Committee for Medical Research Ethics (2013/265 REK, Norway). Fourteen tissue sections of human articular cartilage were obtained from the femoral condyle of 14 different osteoarthritic patients undergoing total replacement knee surgery (Table 1). In order to consider the case of primary OA, only those patients that had not suffered previous knee injury and had not undergone prior surgery, were included in the study. To reduce age-associated variation in the articular cartilage, all patients selected for this study were older than 65 years. The grading of OA was

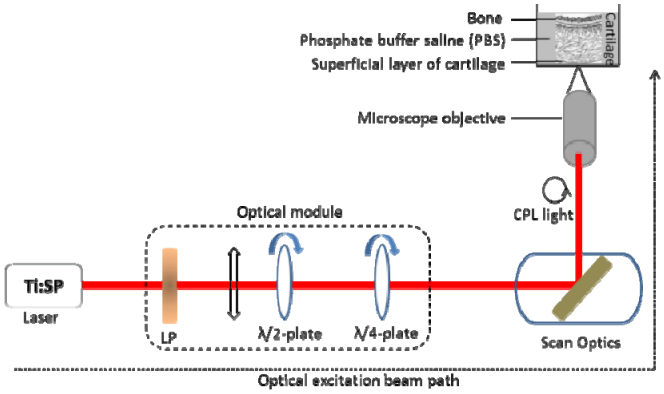

Fig. 1. A schematic diagram showing the optical module inserted in the excitation beam path to achieve CPL for uniform illumination of cartilage. It also shows the orientation of cartilage during acquisition of NLOM image.

based on the standard International Cartilage Repair Society (ICRS) classification [24]. The assignment of ICRS grade to the samples was performed by two experienced orthopedic surgeons, who were blinded to the classification of each other. Only samples assigned a similar ICRS grade by both orthopedic surgeons were included in this study. Since this study focus on investigation of early stage osteoarthritic cartilage using NLOM microscopy, only ICRS Grade-I osteoarthritic cartilage samples were included. The cartilage samples were dissected with a surgical scalpel, perpendicular to the articular surface (from superficial layer to the bone), fixed in formalin and stored at 4°C. During the measurement, to avoid dehydration of the tissue during imaging, the sample was placed in an imaging chamber filled with PBS. The sample was placed on the bottom of the chamber (Fig. 1) in such a way that superficial layer of cartilage was facing the microscope objective (40X, 0.8NA).

Microscopy images were acquired using a commercial Zeiss LSM 510 META microscope. For the excitation of the tissue sample 200 fs laser pulses with a repetition rate of

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76 MHz was tuned to 790 nm. A combination of dichroic mirror and short pass filter was used to reject backscattered laser light while a band pass filter (395 ± 25 nm) and a long pass filter was used to select SHG and TPEF signal, respectively. In order to illuminate the collagen structures uniformly, the cartilage sample was excited by circularly polarized light (CPL). CPL light in the sample plane was produced by introducing an additional optical module (Fig. 1) in the optical path of the microscope [25]. SHG and TPEF images covering areas of 225µm × 225µm were selected in the cartilage and the signal (256 gray levels) was recorded in 512 pixels × 512 pixels matrices.

3. Results and discussions

One of the major pathological changes of OA is fibrillation followed by progressive thinning and loss of articular cartilage. In general, morphological information obtained by histological evaluation is considered adequate to define the pathological state of the degenerated cartilage. Surface erosion and appearance of fissure in cartilage are two of the major histological features [26]. However such features are prominently visible only in advanced stages of OA. A typical histology (hematoxylin and eosin stained) and arthroscopy view of early stage (ICRS Grade-I) osteoarthritic cartilage is shown in Fig. 2. A careful view indicates the presence of slight fibrillation/erosion on the cartilage surface. In several OA model systems, for early and even mild phase of the disease, histological and arthroscopic, both grading systems are observer dependent [27–29]. The reproducibility and the validity of these grading systems have been questioned [27–29].

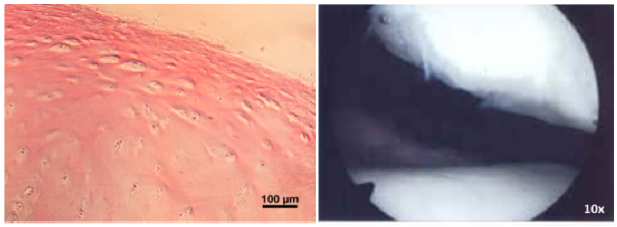

Fig. 2. Histology (H&E stain) and arthroscopy image of a typical ICRS Grade-I osteoarthritic cartilage.

Within the group of ICRS Grade-I osteoarthritic cartilage, distinct features were observed by SHG microscopy. 9 specimens (id: # 1,2,4,5,8,9,11,12,13, see Table 1) showed splits on the surface of the cartilage (Fig. 3). The size of the observed splits was approximately 1-2 µm in width and 10-80 µm in length (Fig. 3). Optical sectioning revealed that the size of these splits gradually decreased with depth from the articular surface and were visible up to a depth of typically ~60 µm (Media 1). A deeper (>60 µm) inside the tissue, due to multiple scattering, the visibility (or contrast) of SHG image was not good enough to recognize the presence of splits. These observed micron size splits (microsplits) are much smaller than the fissures which are observed in histology. Since, the smallest width of observed microsplit (~1-2 µm) is near to the resolution limit of optical microscope. The presence of splits at submicron scale might be possible at higher depth. Hence, further investigation by electron microscopy may provide more information about the existence of splits whose size is beyond the level of optical resolution. However, in case of electron microscopy, essential tissue processing (e.g., embedding, dehydration, staining etc.) may limit such investigation.

The presence of microsplits can resist the smooth movement of the articulating surfaces in a knee joint during motion. Thus, it may enhance the friction between the two cartilage surfaces within a synovial joint. Due to unavailability of pain receptors in cartilage, any enhancement in the friction between the two cartilage surfaces cannot be felt during the movement and is thus not symptomatic. In the long term, it can further amplify the degenerative process of articular cartilage. Therefore, the occurrence of microsplits might

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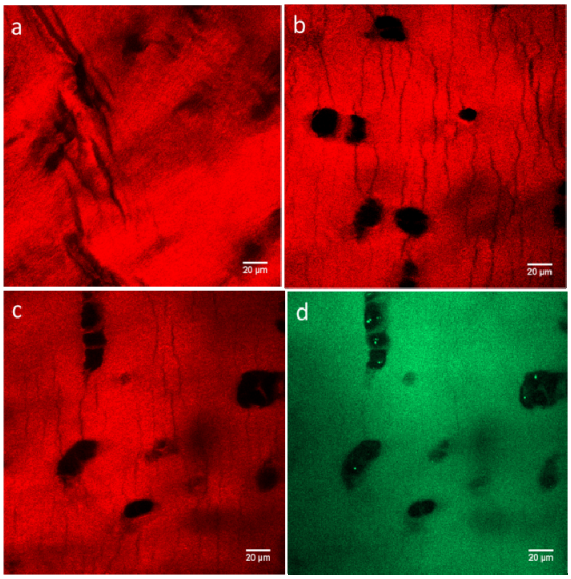

Fig. 3. SHG images of ICRS Grade-I osteoarthritic cartilage that shows microsplits at depth (a) 5µm (b) 15µm and (c) 30µm from the articular surface of cartilage. (d) TPEF image of the section 3(c). The TPEF image does not show microsplits as clear as in SHG image.

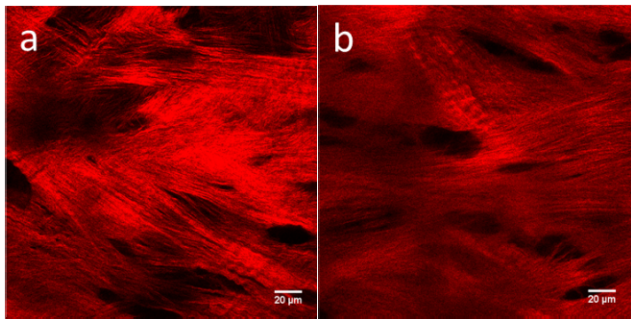

Fig. 4. SHG images of ICRS Grade-I osteoarthritic cartilage that shows superficial layer at depth (a) 5µm (b) 15µm.

make the cartilage more vulnerable. It has to be noted that this feature is not visible in arthroscopy or any other clinical modality. Even the TPEF image (Fig. 3(d)) of the same section was not able to show the microsplits as clearly as in the SHG image. This may be due to the presence of other extracellular components in the microsplits that gives auto-fluorescence and mask the microsplits. Due to high molecular specificity towards collagen and high resolution, such microsplits are visible only in SHG microscopy. Previously, a similar kind of microsplit was observed in an induced osteoarthritic model [17].

Four specimens (id: # 3,7,10,14, see Table 1) showed different features compared to the 9 specimens that showed the presence of microsplits (Fig. 4 and Media 2). In these four specimens the arrangement of collagen fibers parallel to the articular surface was observed up to a typical depth of ~70 µm from the articular surface of cartilage. Such arrangement of collagen fibers indicate the presence of a superficial region of cartilage surface, which is a feature of normal (healthy) cartilage [30]. Due to the presence of large diameter of individual fiber, a fibrilar- like structure is generally visible in the superficial layer of articular cartilage.

Based on our observations, we hypothesized that these two morphological features (occurrence of microsplit and presence of collagen fiber parallel to articular surface) represent two different phases or stages of cartilage degradation during progression of OA. Initially, a

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superficial layer (i.e., larger fibrillar size collagen fiber) is present, but as OA progresses it gradually disappears due to erosion and thus, only the middle to deep regions of cartilage are left. This affect the biomechanics of cartilage in knee joint and therefore, an imbalance in resistance of shear forces may create microsplits in the surface of articular cartilage.

In 3 specimens (id: # 5,9,13, see Table 1) in addition to microsplits, another feature consisting of wrinkle like structure was also observed (Fig. 5 and Media 3). The appearance of a wrinkle-like structure indicates the loosening of cartilage architecture and integrity. The frictional forces of the opposing cartilage surface, during movement of the knee joint, may start to displace the cartilage surface causing appearance of wrinkled structure. Note that this feature was not clearly visible in corresponding TPEF image (Fig. 5(d)), which indicates that this abnormality is associated mainly with collagen fibers. Similar feature have been reported in the center of a lesion in an advanced degenerated cartilage obtained from a horse [12]. The elevation and loosening of the articular cartilage especially collapse of collagen network in the weight bearing area followed by fibrillation and thinning of the remaining area was reported as a characteristic feature in osteoarthritis [31–33]. Our observations have two indications. First, the wrinkling is not only present in load bearing area (medial) but also in non-load bearing area (lateral) of the knee. Second, wrinkling is not only found in advanced stage of OA but also present in early stage of OA and therefore may serve as an early indicator of cartilage degeneration.

The progression of OA is characterized by several disorders (e.g., change in ECM composition and structure, change in cellular activity) that can lead to degradation of cartilage as well as to an inflammatory states. Chondrocytes are able to adapt their metabolic response to changes in the physical environment. In the present investigation we were mainly interested in morphological feature of early OA especially the change in collagen network of articular cartilage.

Using NLOM microscopy, we successfully demonstrated various morphological features, which appeared due to early stage modification in the extracellular matrix (ECM) of osteoarthritic cartilage. These features typically would not be visible in other clinical imaging modalities especially in early stage (low grade) of OA. Within the stage of ICRS Grade-I, different morphological features were observed, which indicates two distinct phases of cartilage degradation that can be distinguished by the use of SHG microscopy. The presence of microsplits and wrinkle like structures on the surface of articular cartilage may help in understanding the underlying mechanism relevant to progression of OA and potentially may serve as early biomarker of OA. In one specimen (id: # 6), although superficial layer was worn out and only middle to deep region of cartilage was visible, no microsplits were observed. This requires further investigation. Due to ethical considerations, biopsies of normal (healthy) cartilage from human knee were not possible to obtain in this study.

In our investigation, a particular attention was given to minimal tissue processing such that any unnecessary induced morphological changes can be avoided. Formalin fixed cartilage sections were imaged without any further processing. Our experience with various other

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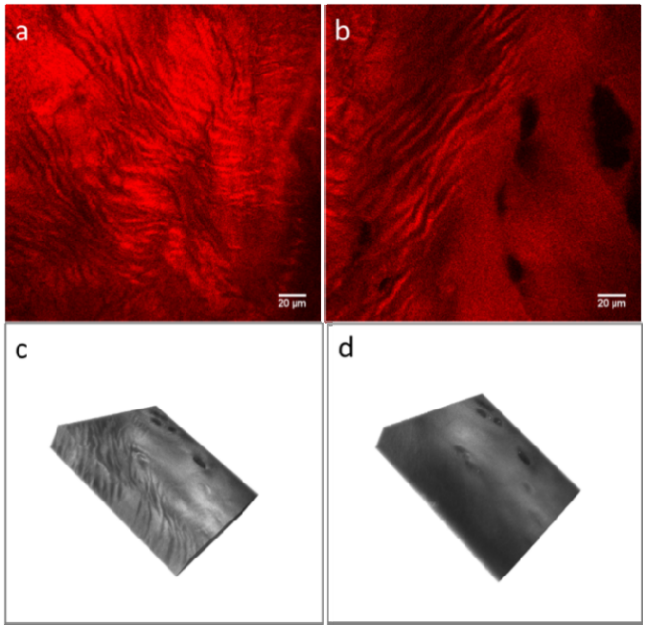

Fig. 5. SHG images of ICRS Grade-I osteoarthritic cartilage that shows ripples/wrinkles at the depth (a) 5µm (b) 15µm. (c) SHG-3D perspective view of rippling. (d) TPEF image of the section 5(c). The feature of rippling is not observed clearly in TPEF image.

Table 1. Morphological features that were observed in ICRS Grade-I osteoarthritic human cartilage

Patient Id #	Tissue location (femoral condyle)	Micro-splits	Superficial layer	Wrinkles
1	Lateral Posterior	X		
2	Lateral Posterior	X		
3	Lateral Posterior		X	
4	Medial Posterior	X		
5	Lateral Anterior	X		X
6	Lateral Posterior			
7	Lateral Posterior		X	
8	Lateral Posterior	X		
9	Medial Posterior	X		X
10	Lateral Posterior		X	
11	Lateral Posterior	X		
12	Lateral Posterior	X		
13	Lateral Posterior	X		X
14	Lateral Posterior		X	

cartilages (e.g., chicken, porcine) showed that formalin fixation had no discernable effect on the SHG image. No physical sectioning of the tissue and any external label was applied in this investigation. Moreover, in order to avoid the limitation of sample thickness, all SHG/TPEF measurements were performed in the backscattered imaging mode. Due to the thickness of tissue sample, forward imaging is not possible. This demonstrates that the technique is compatible with *in vivo* investigation by the use of a miniaturized NLOM probe, which necessarily uses backscattered imaging. An endoscopic LOM probe is under development by several groups [20–22].

4. Conclusion

The observations in this study demonstrate the unique advantage of using NLOM, especially SHG microscopy to image articular cartilage. In summary, our proof-of-principle

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investigation present SHG microscopy as a potential tool which, with high specificity and sensitivity, can assess the quality of articular cartilage and therefore may be able to identify the stage of OA more precisely than other existing clinical modalities. Our pilot study encourages further investigation on a large sample population to validate the novel structural and morphological features that may ultimately serve as biomarkers in early diagnosis of OA.

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