

1           Ultrasound Biomicroscopy Imaging for Monitoring Progressive Trypsin  
2                           Digestion and Inhibition in Articular Cartilage

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9   Running Title: Ultrasonic Monitoring of Cartilage Trypsin Digestion

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1 **Abstract**

2 This study reports an ultrasound biomicroscopy (UBM) imaging approach to monitor the  
3 progressive trypsin-induced depletion of proteoglycan (PG) and its inhibition in articular  
4 cartilage. Three fresh, normal bovine patellae were obtained, and four full-thickness  
5 cartilage-bone specimens were prepared from the lower medial side of each patella. One  
6 sample was used as a control and the other three were divided into three groups: Groups A, B,  
7 and C (n=3 for each group). After a 40 min 0.25% trypsin digestion, samples from Group A  
8 were continuously digested in trypsin solution, while those in Groups B and C were immersed  
9 in physiological saline and fetal bovine serum (FBS), respectively, for another 280 min. The  
10 trypsin penetration front was observed by UBM, and M-mode images were acquired using 50  
11 MHz focused ultrasound and custom-developed software. The results show that the 40 min  
12 trypsin digestion degraded nearly the whole surface layer of the cartilage tissue. Further  
13 digestion in trypsin or residual digestion in saline for 280 min depleted most of the PG content,  
14 as observed in Groups A and B. The replacement of trypsin with a physiological saline  
15 solution only slightly slowed the digestion process (Group B), while trypsin inhibitors in FBS  
16 stopped the digestion in approximately 1.5 h (Group C). The normalized digestion fractions of  
17 the digested tissues were calculated from ultrasound data and histology sections, and then  
18 compared between the groups. Without the use of FBS, 80-100% of the full thickness was  
19 digested, while this number was only approximately 50% when using FBS. Our findings  
20 indicate that the UBM imaging system could provide 2-D visual information for monitoring  
21 progressive trypsin-induced PG depletion in articular cartilage. The system also potentially  
22 offers a useful tool for preparing cartilage degeneration models with precisely controlled PG  
23 depletion.

24 **Keywords:** Articular cartilage; Ultrasound; High frequency ultrasound; Ultrasound  
25 biomicroscopy; Proteoglycan depletion; Trypsin digestion; Trypsin inhibitors; Osteoarthritis

26

27

## 1 INTRODUCTION

2 Articular cartilage (artC) is a low frictional, load-bearing soft tissue that provides almost  
3 frictionless support for several joints. The material composition of artC is a multi-phase  
4 hydrated mixture mainly composed of 5–10% proteoglycan (PG), 10–20% collagen, and  
5 60–80% water (Mow et al. 2005). Aggregating PGs are negatively charged  
6 bio-macromolecules that are enmeshed in the collagen matrix (Lai et al. 1991; Maroudas  
7 1976). Therefore, PGs play an important role in determining the electrochemical and  
8 mechanical properties of articular cartilage, such as shear modulus (Zhu et al. 1993),  
9 compressive modulus (Qin et al. 2002; Zheng et al. 2001, 2005), swelling strain (Narmoneva  
10 et al. 1999; Wang and Zheng 2006) and swelling aggregate modulus (Flahiff et al. 2004;  
11 Narmoneva et al. 2002; Wang et al. 2007, 2008a). It has been widely reported that the loss of  
12 PGs is one of the earliest signs of osteoarthritis (OA), which is one of the most common joint  
13 diseases. Advanced stage OA is characterized by a partial or total loss of cartilage tissue and  
14 the direct contact of bone across the joint. (Armstrong and Mow 1982; Martini 2004; Sandy  
15 2003; Torzilli et al. 1990). Early diagnosis of artC degeneration is very important for the  
16 treatment of OA. However, conventional methods using X-ray imaging can only detect very  
17 late stage OA. Most existing MRI systems do not have sufficient resolution for the assessment  
18 of artC. Conventional ultrasound scanners are not suited to the direct study of OA, as their  
19 resolution is also usually insufficient to image changes in artC. Further, it is difficult to scan  
20 the whole articulating surface with an ultrasound probe located outside the body. New  
21 approaches using arthroscopic imaging can provide more information about artC degeneration  
22 processes such as surface fibrillation, but it remains challenging to assess early stage OA

1 using video inspection. In order to evaluate new therapies such as mosaic plasty, cultured  
2 chondrocyte transplantation, and even the use of tissue-engineered cartilage, it is necessary to  
3 develop sensitive assays of the therapeutic outcome (Detterline et al. 2005; Fu et al. 2003).  
4 During the last decade, various techniques have been developed for this purpose, including  
5 mechanical indentation (Toyras et al. 2001), quantitative ultrasound (Saied et al. 1997; Joiner  
6 et al. 2001; Hattori et al. 2003; Nieminen et al. 2002, ), ultrasound combined with indentation  
7 (Zheng and Mak 1996; Suh et al. 2001; Laasanen et al. 2002), water-jet indentation (Lu et al.  
8 2009), compression (Zheng et al. 2004a), osmotic loading (Wang et al. 2008a), optical  
9 coherence tomography (OCT) (Herrmann et al. 1999), air-jet OCT indentation (Huang et al.  
10 2009), and electromechanical measurement (Legare A et al. 2002).

11

12 Various in-vitro and in-vivo animal models mimicking human articular cartilage degeneration  
13 have been reported in the literature. These models are intended to aid the development of new  
14 diagnostic approaches and the investigation of the mechanism of degeneration. To study how  
15 the loss of PGs results in changes in morphological, biomechanical, acoustic, and bioelectrical  
16 parameters, many studies have treated normal articular cartilage with enzymes such as trypsin.  
17 Such treatment depletes PGs, mimicking natural cartilage degeneration (Basser et al., 1998;  
18 Brown et al., 2007; Deng et al., 2007; Disilvestro and Suh, 2002; Harris et al., 1972; Hunziker  
19 and Rosbenberg 1996; Laasanen et al., 2002; Moody et al., 2006; Nieminen et al., 2002; Qin  
20 et al., 2002; Saarakkala et al., 2004; Suh et al., 2001; Toyras et al., 2002; Zheng et al., 2001,  
21 2004). In terms of the understanding of how the loss of PGs affects different physical  
22 parameters of cartilage, tremendous achievements have been made using these trypsin-treated

1 animal and human cartilage models. In spite of these advances, one fundamental problem in  
2 preparing these models has not been solved: how to quantify the degree of PG depletion  
3 during the trypsin treatment. In other words, how can we prepare a cartilage degeneration  
4 model with exactly the required degree of PG depletion?

5

6 The conventional way to quantify PGs in cartilage is to use histological assays by binding  
7 different stains, such as safranin-O, toluidine blue, hematoxylin and eosin (H&E), and alcian  
8 blue, to differentiate different compositions in cartilage (Lyons et al. 2006). However, this  
9 method is time-consuming and invasive since it involves specimen collection, fixing, staining,  
10 slicing, imaging, and analyzing. In many studies, pilot tests are used to determine the degree  
11 of PG depletion under a certain concentration of trypsin, operating temperature, and period of  
12 treatment. Since variations in the cartilage thickness and in the distribution of PGs and  
13 chondrocytes are commonly observed between different specimens (Moody et al. 2006), it is  
14 difficult to control the trypsin digestion process using histological techniques to create  
15 calibration standards. An alternative method is to conduct histological examination after  
16 completing all necessary (nondestructive) tests on the trypsin-treated cartilage specimen, so as  
17 to quantify the PG depletion level of each specimen. This is time-consuming and, in many  
18 cases, the histologically examined PG depletion level may not be the level expected for the  
19 experimental design. Unfortunately, at that point, it is too late to alter the experiment based on  
20 this knowledge. Therefore, it is very important to have a real-time monitoring approach for  
21 preparing cartilage degeneration models with a defined PG depletion level. Furthermore,  
22 enzyme inhibitors such as fetal bovine serum (FBS) are commonly used to stop trypsin

1 digestion at a certain PG depletion level (Xiang et al., 2006). Using routinely available  
2 methods, it is not possible to determine precisely how long it takes for FBS to stop the trypsin  
3 digestion process or how the inhibition process proceeds, though it has been estimated in  
4 previous studies that a FBS solution can stop trypsin digestion within approximately one hour  
5 (Qin et al., 2002; Rieppo et al., 2003). In addition, although washing with saline after trypsin  
6 treatment is a very commonly used technique in the literature, we do not know whether  
7 replacing the trypsin solution with a saline solution can stop the digestion process.

8

9 Inspired by the reduction of elasticity of articular cartilage with depletion of PGs, a novel  
10 technique using high frequency ultrasound to monitor the process of trypsin digestion has  
11 been recently reported (Toyras et al., 2002; Nieminen et al. 2002; Zheng et al. 2004b; Wang et  
12 al. 2008b). The fundamental principle of this technique is that the reduction in the elasticity of  
13 PG-depleted cartilage leads to a decrease in its acoustic impedance and, thus, creates an  
14 artificial acoustic interface between the digested and undigested cartilage tissues. An  
15 ultrasound echo can be generated at this interface, and this echo shifts as the trypsin digestion  
16 progresses deeper into the tissue. With continuous recording of ultrasound signals, the trace of  
17 digestion can be viewed in an M-mode ultrasound image. This novel technique allows  
18 real-time monitoring of the process of PG depletion induced by trypsin digestion, though the  
19 reported works are still very preliminary. These earlier studies were mainly focused on the  
20 feasibility of this technique (Toyras et al., 2002; Nieminen et al. 2002; Zheng et al. 2004b).  
21 The digestion speed of a certain concentration of trypsin in cartilage was recently reported  
22 (Wang et al. 2008b). In these studies, the ultrasound beam was focused at a single location in

1 the articular cartilage. Thus, it is difficult to get an accurate digestion profile and it is not  
2 known whether the digestion process is site-sensitive. Furthermore, no study has been  
3 reported that monitors the trypsin inhibition process, an important step in preparing OA  
4 models with different degeneration levels.

5

6 In the present study, we test the feasibility of ultrasound biomicroscopy (UBM) imaging, or  
7 “acoustic microscopy” (Foster et al., 2000; Saijo and Chubachi 2000) for monitoring the  
8 progressive trypsin digestion process along the digestion front, as well as the inhibition  
9 process of FBS against trypsin. To investigate the inhibition efficiency of FBS against trypsin,  
10 we also examined residual trypsin digestion in physiological saline. Instead of monitoring the  
11 cartilage tissue at a single location as was done in earlier works, UBM provides a real-time,  
12 continuous view of the digestion front on the entire cross-section of the cartilage. The results  
13 obtained in this study could help in understanding the interaction between trypsin and its  
14 inhibitors. If proved feasible, UBM imaging would qualify as a useful tool for future studies  
15 involving the examination of the diffusion of different solutes, including enzymes and their  
16 inhibitors, in articular cartilage.

17

## 18 **MATERIALS AND METHODS**

### 19 *Specimen preparation*

20 Three fresh mature bovine patellae without apparent lesions were obtained from a local  
21 butcher shop within 6 hours of slaughter, and were stored at -20°C until further preparation.  
22 For each patella, four cartilage-bone plugs were prepared from the lower medial side using a

1 metallic punch with a diameter of 6.35 mm (Fig. 1). One of these samples was be used as a  
2 control, and the other three divided into three groups (A, B, and C) for trypsin treatment. The  
3 experimental protocols for each group are described in subsequent sections. The prepared  
4 cartilage-bone plugs were wrapped in wet gauze soaked with physiological saline and stored  
5 at -20°C until the experiment.

6

#### 7 *The ultrasound biomicroscopy (UBM) system*

8 The ultrasound signals backscattered from the cartilage sample were acquired using a UBM  
9 system similar to the ultrasound system described in our previous reports (Wang and Zheng,  
10 2006; Zheng et al., 2006). The UBM system, as shown in Fig. 2a, comprised a nominal 50  
11 MHz focused ultrasound transducer (Model PI50-2, Panametrics, Waltham, MA, USA), a  
12 pulser/receiver (Model 5601A, Panametrics, Waltham, MA, USA), a 500 MHz 8-bit A/D  
13 converter (Model CompuScope 8500PCI, Gage, Canada), and a computer with  
14 custom-designed software. The transducer had a focal length of 12.7 mm, a diameter of 6.35  
15 mm, a -6 dB beamwidth of 0.1 mm, and a -6 dB focal zone depth of 0.95 mm. The system  
16 was equipped with a 3-D translating device (Parker Hannifin Corporation, Irvine, CA, USA),  
17 which was used to adjust the position of the transducer and to drive the transducer to scan the  
18 specimen. The scan length was 6.8 mm with a displacement step of 50 µm along the specimen  
19 diameter. These conditions allowed acquisition of 2-D UBM images of the tissue in  
20 approximately 60 sec (Fig. 2). The UBM images displayed the echoes reflected from the  
21 saline-cartilage interface and the cartilage-bone interface, as well as those scattered from the  
22 cartilage tissue (Fig. 2b). Each UBM image was formed by 137 lines of A-mode ultrasound



1 signals, the amplitude of which corresponded to the grey levels in the image. The baseline of  
2 the radiofrequency (RF) ultrasound signal, i.e., zero magnitude, corresponded to grey level  
3 128 out of 256. A brighter pixel in the image represents a more positive magnitude and a  
4 darker pixel represents a more negative magnitude. Fig. 3 shows the interface of the  
5 custom-developed software, in which a UBM image, the A-mode ultrasound RF signals, and  
6 an M-mode image are simultaneously displayed. The assignment of grey levels for different  
7 signal magnitudes was the same as that used for forming the UMB image.

8

#### 9 *Ultrasound examination*

10 Before testing, the frozen cartilage-bone specimen was thawed in physiological saline solution  
11 (0.15 M NaCl) for three hours at room temperature ( $25\pm 1^\circ\text{C}$ ). The sample was then installed  
12 in the container, and the outer ring of the cartilage surface was gently covered using rubber  
13 gel (Blu-Tack, Thomastown, Australia). This gel allowed trypsin or serum to penetrate into  
14 the sample only from the surface layer toward the cartilage-bone interface (Fig. 2a).

15

16 After the specimen equilibrated in physiological saline, ultrasound examination was  
17 performed in two phases. In the first phase, the saline solution was removed from the  
18 container using an injection syringe and quickly (within 30 seconds) replaced with the trypsin  
19 solution (0.25%, Cat. No. 25200-056, GibCO, Invitrogen Inc. Burlington, ON, Canada). The  
20 progressive digestion of all specimens from Groups A, B, and C was monitored by the UBM  
21 system for 40 minutes. In the second phase, the specimens of Group A were continuously  
22 immersed in the trypsin solution, while the trypsin solution in Group B and Group C was

1 removed within 30 seconds and replaced with physiological saline and fetal bovine serum  
2 (Cat. No. 16000-036, Gibco, Invitrogen Inc. Grand Island, NY, USA), respectively. We  
3 refer to these two processes as the residual trypsin digestion in physiological saline and the  
4 inhibition in FBS. This phase lasted for 280 minutes.

5

6 In M-mode images, markers were manually inserted at an interval of approximately 5 min  
7 along the inclined trace (see Fig. 3 M-mode Display). This trace represents the process of  
8 trypsin degradation of the PGs, or the interaction between trypsin and its inhibitors.  
9 According to the locations of these markers, the software automatically interpolates the curve  
10 representing the depth of trypsin digestion as a function of time. This procedure was repeated  
11 six times for each specimen at different locations along the center of the 2-D cross-sectional  
12 image to obtain 6 different interpolation curves. These curves were then averaged to reduce  
13 possible site variance of the digestion and to smooth the trace. The variation among the 6  
14 digestion traces of each individual specimen was tested by calculating the root mean square  
15 (RMS) difference with respect to the averaged trace. The average curve was used for  
16 estimation of the digestion fraction (the digestion depth normalized to the cartilage  
17 full-thickness). These normalized values were used for comparison between the ultrasonic and  
18 histological measurements, as the normalization can reduce effects caused by variations of  
19 ultrasound speed between different cartilage samples and potential shrinkage of the specimen  
20 during the process of histological analysis. The averaged digestion traces obtained for the 3  
21 individual samples for each group were further averaged, and their RMS differences were also  
22 calculated, to examine the variation among samples.

1

2 In this study, the full thickness of the cartilage layer and the digestion depth were calculated  
3 using a previously described method (Zheng et al., 2002). The cartilage thickness  
4 measurement was based on the estimation of the time-of-flight between the echoes reflected  
5 from the cartilage surface and the cartilage-bone interface. A cross-correlation technique was  
6 used to match echoes. Since the use of a constant sound speed in both healthy and  
7 PG-depleted cartilage causes an overestimation in the measurement of cartilage thickness  
8 (Pellaumail et al., 2002), sound speeds of 1675 m/s and 1668 m/s were taken for the normal  
9 and PG-depleted cartilage tissues, respectively (Nieminen et al., 2002; Patil et al., 2004).

10

### 11 *Histology*

12 After ultrasound examination, all control and trypsin-treated specimens were fixed in a  
13 buffered formalin solution (10%), decalcified in a 10% EDTA solution using an ultrasound  
14 method reported by Guo et al. (2007), then embedded in paraffin. The central region of the  
15 specimen was serially sectioned using a rotary microtome (Leica RM-2135, Cambridge, UK).  
16 The 4 µm-thick deparaffined sections were stained with safranin O (Cat. No. F7258, SiGMA,  
17 MO, USA) and contra-stained with fast green (Cat. No. S-2255, SiGMA, MO, USA). The  
18 sections were viewed with an imaging system comprising a microscope (Model FN-S2N,  
19 Nikon, Japan) and a digital camera (DXM 1200X, Nikon, Japan). In the optical micrographs,  
20 red color due to safranin O indicates the presence of PG (Leung et al. 1999; Qin et al. 2002).  
21 The depth of the trypsin-digested zone was estimated by measuring the depth of the fast  
22 green-stained zone in the histological image. To obtain the digested fraction, the digestion

1 depth was normalized by the full cartilage thickness, measured from the cartilage surface to  
2 the interface between the deep cartilage layer and the bone. The 3 digestion fraction values  
3 obtained from 3 sections for each specimen were averaged, and the mean value was compared  
4 with the ultrasound measurement.

5

## 6 **RESULTS**

7 Fig. 4 shows UBM images at the central sample section collected after 0 min, 40 min, 2 h, and  
8 4 h of trypsin digestion of three cartilage samples from Groups A, B and C. As the trypsin  
9 penetrated into the cartilage tissue and broke down the aggregating PGs, an interface between  
10 the digested and undigested tissues was generated due to the change in the acoustic impedance  
11 of the PG-depleted tissue. Consequently, an additional ultrasound echo appeared. As digestion  
12 continued, this interface moved deeper into the tissue (Fig. 4). In the first phase of this study,  
13 we observed that the 40 min trypsin treatment digested approximately 30-40% of the full  
14 thickness of the cartilage (Fig. 4b, f, j). In the second phase, the digestion of Group A  
15 continued until, after 2 h of trypsin digestion, the interface was close to the bone (Fig. 4c).  
16 The digestion front reached the bone and disappeared after 4 h of digestion (Fig. 4d). For  
17 Group B, the digestion front continued to shift down into the deep layer even after trypsin was  
18 replaced with physiological saline (Fig. 4g, h). In Group C, trypsin was replaced with FBS,  
19 which contains trypsin inhibitors. Although this replacement is supposed to stop trypsin  
20 digestion, the digestion front (Fig. 4k) kept moving deeper after 2 h of inhibition, as compared  
21 to the front in Fig. 4j. Between 2 h and 4 h of inhibition (Fig. 4k and Fig. 4l), the digestion  
22 front entered a static phase and moved little. As seen in Fig. 4, the digestion level of Groups A

1 and B was much deeper than that of Group C.

2

3 The results demonstrate that the digestion trace extracted from the M-mode image was rather  
4 repeatable among the 6 traces for a single specimen, and among the traces of the three  
5 specimens in each group. The averaged root mean square (RMS) variations (with respect to  
6 the corresponding averaged traces) were  $5.1\pm 1.5\%$  for a single specimen and  $7.4\pm 3.0\%$   
7 among the three specimens. By analyzing the inclined digestion traces in the M-mode images  
8 from different groups (Fig. 5), we found that the pattern of residual trypsin digestion  
9 experienced by Group B was similar to the trypsin digestion pattern of Group A, but different  
10 from the inhibition process of Group C. In the second phase of the study, i.e., after the first 40  
11 min trypsin digestion, we measured the digestion depth and plotted it as a function of time for  
12 the three groups (Fig. 6). Since no more trypsin entered into the tissue, the residual digestion  
13 of Group B in physiological saline slowed down in comparison to the continuous trypsin  
14 digestion in Group A. Approximately 0.8 mm and 0.6 mm of cartilage were digested after 280  
15 min for groups A and B, respectively. Group C underwent a digestion process similar to  
16 Groups A and B during the first 50 min, when the trypsin inhibitors might have not penetrated  
17 into the tissue. After that time, the inhibitors started to affect the function of trypsin. This  
18 inhibiting process lasted for approximately one hour (Fig. 6), until the movement of the  
19 digestion front stopped. The final digestion depth for Group C was approximately 0.3 mm.

20

21 We calculated the digestion fractions from histological sections of the specimens in the  
22 control group and groups A, B, and C and compared these with the results of the ultrasonic

1 measurements (Fig. 5, Table 1). The results reveal that all or almost all of the PGs in the entire  
2 cartilage layer were digested by the 280-minute trypsin digestion and by the residual digestion.  
3 FBS effectively stopped trypsin digestion at the middle layer, while physiological saline only  
4 slightly weakened the trypsin digestion in the cartilage. According to Table 1, the results of  
5 the ultrasonic and histological measurements on the digested portion of the trypsin-treated  
6 articular cartilage did not agree well for specimens treated only by trypsin, or for those treated  
7 with trypsin and serum. The results of the specimens treated with trypsin and saline did agree  
8 well.

9

## 10 **DISCUSSION**

11 In this study, we observed in real-time the penetration of trypsin digestion and the process of  
12 FBS inhibition using UBM cross-sectional images as well as M-mode ultrasound images. We  
13 performed both imaging techniques by detecting the additional echoes reflected from the  
14 interface between the digested and undigested tissues. The enzyme digestion front moved  
15 vertically from the surface layer to the deeper layer with long digestion times. Among the  
16 three specimen groups tested, the overall process of trypsin digestion was similar within the  
17 first 40 min. Further digestion in trypsin, residual digestion in saline, and inhibition of  
18 digestion in FBS gave consistent results within the 3 specimens tested for each group, with an  
19 RMS deviation of  $7.4\pm 3.0\%$ . However, we observed differences between the specimens in the  
20 profile of the digestion front, the magnitude of the interface echo, and the depth of the  
21 digestion front at a given time. This finding agrees with the results of a recent study that also  
22 reported variations in trypsin digestion between different specimens (Moody et al., 2006).

1 Most importantly and interestingly, we found that the digestion front in the tissue section was  
2 not a straight line. This finding indicates that either the distribution of the PGs or the diffusion  
3 rate of trypsin along the depth direction varied from site to site, even in a cartilage disc with a  
4 diameter of only 6.35 mm. Our histological images also showed that the interfaces between  
5 the safranin O-stained and fast green-stained areas were not exactly horizontal for all  
6 specimens. This inhomogeneity of trypsin digestion further demonstrates the inhomogeneous  
7 distribution of PGs and other components in cartilage tissue. The results also confirm the  
8 necessity of using 2-D imaging to monitor the digestion and inhibition process, as the  
9 single-location monitoring reported in earlier studies may not give a complete description of  
10 the digestion front (Toyras et al., 2002; Nieminen et al. 2002; Zheng et al. 2004b; Wang et al.  
11 2008). Although the sides of the cartilage specimens were sealed with a rubber gel, it might be  
12 still possible that the trypsin solution diffused into the cartilage tissue from the sides. This  
13 possibility may explain why the interface echo was relatively weak or disappeared towards the  
14 two sides of the cross-sectional image, as observed in the images of Fig. 4. Further studies  
15 may be warranted to systematically investigate the lateral diffusion of trypsin solution from  
16 the sides of specimens. 3-D ultrasound imaging of articular cartilage may provide even more  
17 insight and direct information about what occurs in the tissue during progressive degeneration.  
18 Early studies have attempted 3-D imaging of articular cartilage with morphological lesions or  
19 topographic changes due to OA (Cashman et al., 2002; Gaddipati et al., 2004; Lefebvre et al.,  
20 1998). Methods have also been developed to attempt to provide 3-D information about effects  
21 of drugs on articular cartilage (Jaffre et al., 2003). However, little work has been done to  
22 provide real-time 3-D imaging of degeneration processes in articular cartilage. Using 3-D

1 ultrasound imaging techniques to display the progressive degeneration of articular cartilage  
2 should be the subject of further study.

3

4 Besides studying the digestion process, we also investigated the inhibiting effect of FBS  
5 against trypsin digestion using UBM in this study. It has been reported that a FBS solution can  
6 stop trypsin digestion within approximately one hour (Qin et al., 2002; Rieppo et al., 2003). In  
7 our study, it took about one and half hours for FBS to totally inhibit digestion at a trypsin  
8 concentration of 0.25%. In addition to the time required for inhibition, we have also reported,  
9 for the first time, the process of FBS inhibition, which is rather consistent among the three  
10 specimens. In the future, it would be very interesting to study what affects the inhibition  
11 process, and what the differences are between articular cartilage tissue from different species.

12

13 Using UBM, we monitored trypsin digestion, residual digestion in physiological saline, and  
14 the inhibition process of FBS, and revealed the differences between the three processes. Our  
15 results show that the 40 min trypsin-digested specimens continued to be digested by residual  
16 enzyme in the tissue. Replacement of the external solution with saline solution appeared to  
17 have little influence on this digestion. Therefore, using enzyme inhibitors is necessary to  
18 minimize the residual enzyme digestion effect (~0.6 mm further penetration in 280 min) to  
19 achieve a more accurate assessment of the degeneration level of the specimens. Use of  
20 inhibitors will also increase the accuracy of subsequent biomechanical or acoustic  
21 measurements. It should be noted that, despite the use of a digestion inhibitor, the digestion  
22 depth increased approximately 0.2 mm (Fig. 6) during the inhibition process of FBS. This



1 result should be taken into account when using FBS as an inhibition solution. It would very  
2 interesting to further investigate the inhibition process of other types of trypsin inhibitor  
3 solutions. The potential of this ultrasound method to monitor the diffusion process of other  
4 solutes in articular cartilage, such as those studied by MRI techniques (Burstein et al., 1993;  
5 Xia et al., 1995; Woertler et al., 2004), could also be explored in future studies. In our earlier  
6 studies, we have demonstrated that the diffusion of salt (NaCl) between cartilage and the  
7 bathing solution can be monitored using real-time ultrasound. This monitoring is  
8 accomplished by measuring changes in the speed of sound and osmotic swelling in cartilage  
9 (Zheng et al. 2004b; Wang and Zheng 2006; Wang et al. 2007, 2008). Using MRI or  
10 radioisotope marking techniques, the target molecules are directly monitored by their MR or  
11 radiation features. In this ultrasound method, the target molecules are indirectly monitored  
12 through changes in the morphological or acoustic features of the tissue induced by the target  
13 molecules during diffusion. If the feasibility of this ultrasonic approach can be more  
14 systematically demonstrated in future studies, it may potentially provide a more accessible  
15 technique for monitoring various solute transport processes in cartilage and, potentially, other  
16 tissues.

17

18 The finding that residual digestion progresses in saline partially explains the disagreement we  
19 noted in an earlier study between the trypsin digestion depths measured by ultrasound and  
20 histology (Wang et al. 2008b). With the residual digestion in saline taken into account in the  
21 calculation of the digested portion, the results obtained in this study by histology and  
22 ultrasound agree very well for all three cartilage specimens ( $0.84 \pm 0.22$  vs.  $0.83 \pm 0.19$ ).

1 However, for the three specimens treated solely by trypsin for 40 + 280 min, the digested  
2 portion measured with histology was larger than that obtained by ultrasound ( $0.96\pm 0.07$  vs.  
3  $0.88\pm 0.06$ ). This difference can still be explained by residual digestion after the termination of  
4 the experiments. Even though the specimen has not been put in saline solution after the  
5 trypsin treatment, the trypsin in the specimen may continue to degrade the sample after the  
6 end of ultrasound monitoring and removal from the trypsin solution. This residual digestion  
7 occurred after the trypsin treatment, but before the histology test. Thus, this digestion  
8 increased the value of the digestion portion measured by histology, but not the value  
9 measured by ultrasound. For the three specimens treated with trypsin followed by FBS  
10 inhibition, the digested portion measured by histology was smaller than that obtained by  
11 ultrasound ( $0.38\pm 0.06$  vs.  $0.50\pm 0.02$ ). One possible reason for this observation might be that  
12 some of the digestion that occurred during the inhibition process (approximately 0.2 mm, see  
13 Fig. 6) could not be detected by histology (safarin O still stained this portion red). This  
14 phenomenon might result if the PG had not been completely depleted by trypsin prior to the  
15 treatment with FBS. Such an incomplete depletion might have already reduced the tissue  
16 elasticity, affecting the acoustic impedance. However, if the fragments were not small enough  
17 to move out of the collagen network, the main components of PG, chondroitin sulphate and  
18 keratan sulphate, could still be stained red by safarin O. This explanation would account for  
19 the observed transition in color intensity (Fig. 6). Due to the extremely long experimental time  
20 for testing each specimen, we could only test 3x3 specimens in this study, and were not able to  
21 conduct any statistical analysis of these results. Future studies with more cartilage specimens  
22 would be required to draw firmer conclusions about these findings. Further efforts with more

1 specimens and other reference methods are also needed to confirm whether the digestion that  
2 occurred after the appearance of FBS could be viewed with safarin O staining in histology.  
3 These efforts would also confirm whether the mechanical/acoustic interface induced by the  
4 partial trypsin digestion matches the safarin O staining interface. Since the digestion front  
5 observed in ultrasound images moves only when PGs are digested deeper in the sample, we  
6 can use this ultrasound method to detect the real interface between PG-depleted and intact  
7 cartilage tissues. This ability is unaffected by whether the specimen is in trypsin, saline or  
8 inhibitor solution, and does not depend on whether the depleted PG can be viewed by safarin  
9 O staining. Furthermore, it has been reported that histological staining might not accurately  
10 reflect the total PG content in articular cartilage (Camplejohn and Allard, 1998). Considering  
11 these points, the presently-reported ultrasound method may provide not only the benefits of  
12 simplicity and real-time measurements, but possibly also increased accuracy in monitoring PG  
13 depletion in articular cartilage.

14

## 15 **CONCLUSIONS**

16 This study provides a useful approach to observe a dynamic digestion process inside cartilage  
17 tissue. We compared the processes of trypsin digestion, residual digestion in physiological  
18 saline, and FBS inhibition of trypsin digestion. The results show that residual digestion in  
19 physiological saline can degrade cartilage nearly as much as continuous trypsin digestion.  
20 Although FBS did stop digestion in approximately one and half hours, the digestion continued  
21 during the first 30 min of this period. Due to the labor-intensive experiments and extremely  
22 long experimental time, we could only test a limited number of specimens in this study. Future

1 studies with more cartilage specimens from different species could lead to more definite  
2 conclusions. This method may be used to prepare in vitro cartilage degeneration models with  
3 precisely controlled PG depletion levels, or to investigate the diffusion of various solutes,  
4 such as various enzymes or chemicals, inside articular cartilage, as long as they can induce a  
5 change in the acoustic impedance of cartilage. Such precisely controlled OA models may  
6 facilitate the development of new diagnostic tools for early degeneration of articular cartilage  
7 and the investigation of the mechanisms of cartilage degeneration.

8

#### 9 **Acknowledgements**

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11 5245/03E, PolyU 5318/05E, PolyU5354/08E) and The Hong Kong Polytechnic University.

12

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17

18

19

1 **Figure Captions**

2

3 Fig. 1 Schematic of specimen preparation and the experimental protocol.

4

5 Fig. 2 (a) Block diagram of the ultrasound biomicroscopy system. (b) A frame of an  
6 ultrasound biomicroscopic (UBM) image of an area 6.8 mm in width. The image height  
7 represents a single-way ultrasound time-of-flight of 2.05  $\mu$ s, which corresponds to 3.3 mm in  
8 cartilage.

9

10 Fig.3 The user interface of the signal processing software. An M-mode image (upper left, with  
11 a scanning time of 60 s), an UBM image (upper right, representing a width of 6.8 mm) and  
12 A-mode ultrasound RF signals (lower, with a scanning depth of 3.3 mm if fully in cartilage) of  
13 articular cartilage during trypsin digestion were displayed in real-time using the  
14 custom-designed program. The grey levels of the M-mode and UBM image linearly represent  
15 the amplitude of the RF signals, with a grey level 128 out of 256 representing zero magnitude  
16 of the RF signal. The black dots in the M-mode image are the manually placed markers used  
17 for interpolation of the inclined digestion trace. The interval between markers was  
18 approximately 5 min. To avoid overlapping the trace, fewer marks than required are indicated  
19 in the figure.

20

21 Fig. 4 UBM images collected from a typical specimen of Groups A (a-d), B (e-h), and C (i-l).  
22 The samples shown in panels a, e, and i were collected at the beginning of trypsin digestion;  
23 the samples in panels b, f, and j were from the end of the 40 min trypsin digestion; the  
24 samples in c, g, and k were collected after specimens were submerged in trypsin,

1 physiological saline, and FBS, respectively, for 2 hours; panels d, h, and l are the UBM  
2 images after specimens were submerged in trypsin, physiological saline, and FBS,  
3 respectively, for 4 hours. In all images, the width was 6.8 mm and the scanning depth was 3.3  
4 mm if fully in cartilage.

5

6 Fig. 5 M-mode ultrasound images and histological sections obtained at the central region of a  
7 typical specimen from Groups A (a), B (b) and C (c). M-mode images were collected during  
8 the 40 min trypsin digestion, the 280 min trypsin digestion, residual digestion in physiological  
9 saline, and the inhibition process in FBS. Solid white arrows indicate the digestion trace.  
10 Histological images on the right and left sides were collected from intact and digested  
11 specimens, respectively. The dashed line indicates the interface of the PG-depleted and  
12 PG-unaaffected cartilage zone. Red staining by safranin O indicates the presence of PGs.

13

14 Fig. 6 Curves of the averaged digestion depth as a function of time for the three groups. After  
15 the replacement of trypsin with FBS, a period of approximately 1.5 hours was needed to  
16 completely stop the trypsin digestion.

17

18

19

20

21 **Table Caption**

22

23 Table 1: The mean digestion fraction of the digested samples measured at the end of the 40  
24 min trypsin digestion and during various 280 min treatments.

25

1  
2

Figure 1  
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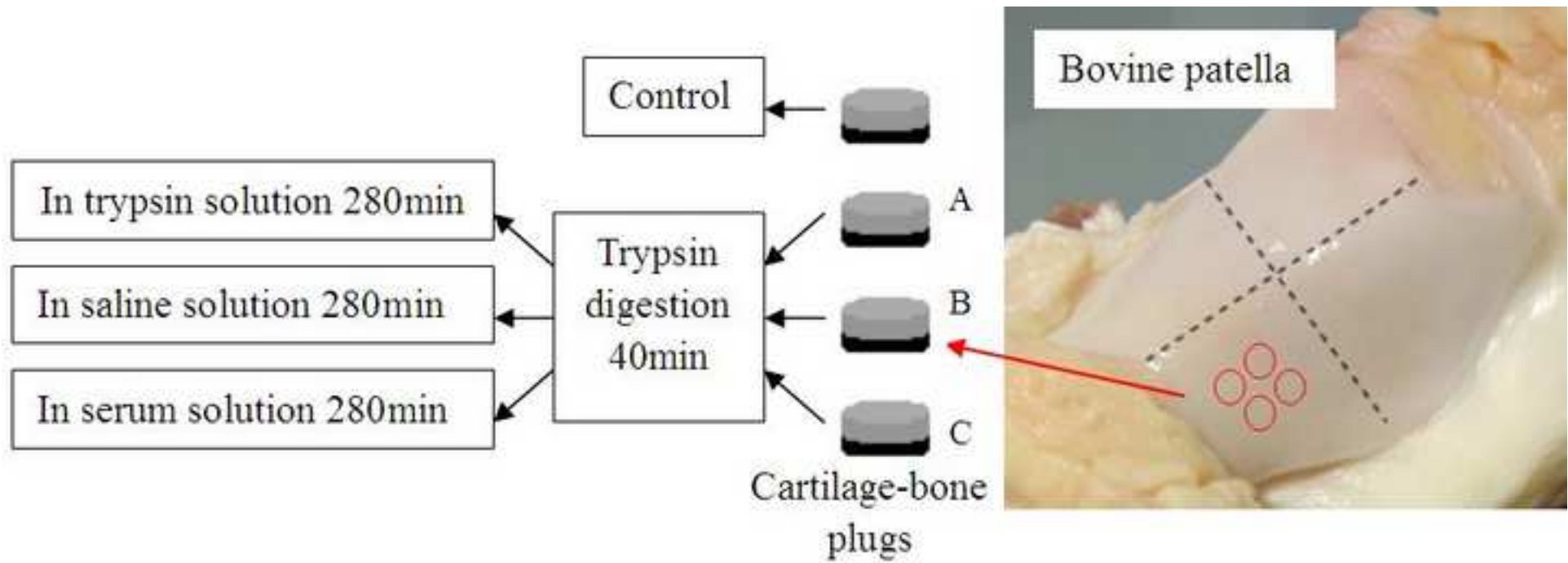


Figure 2a

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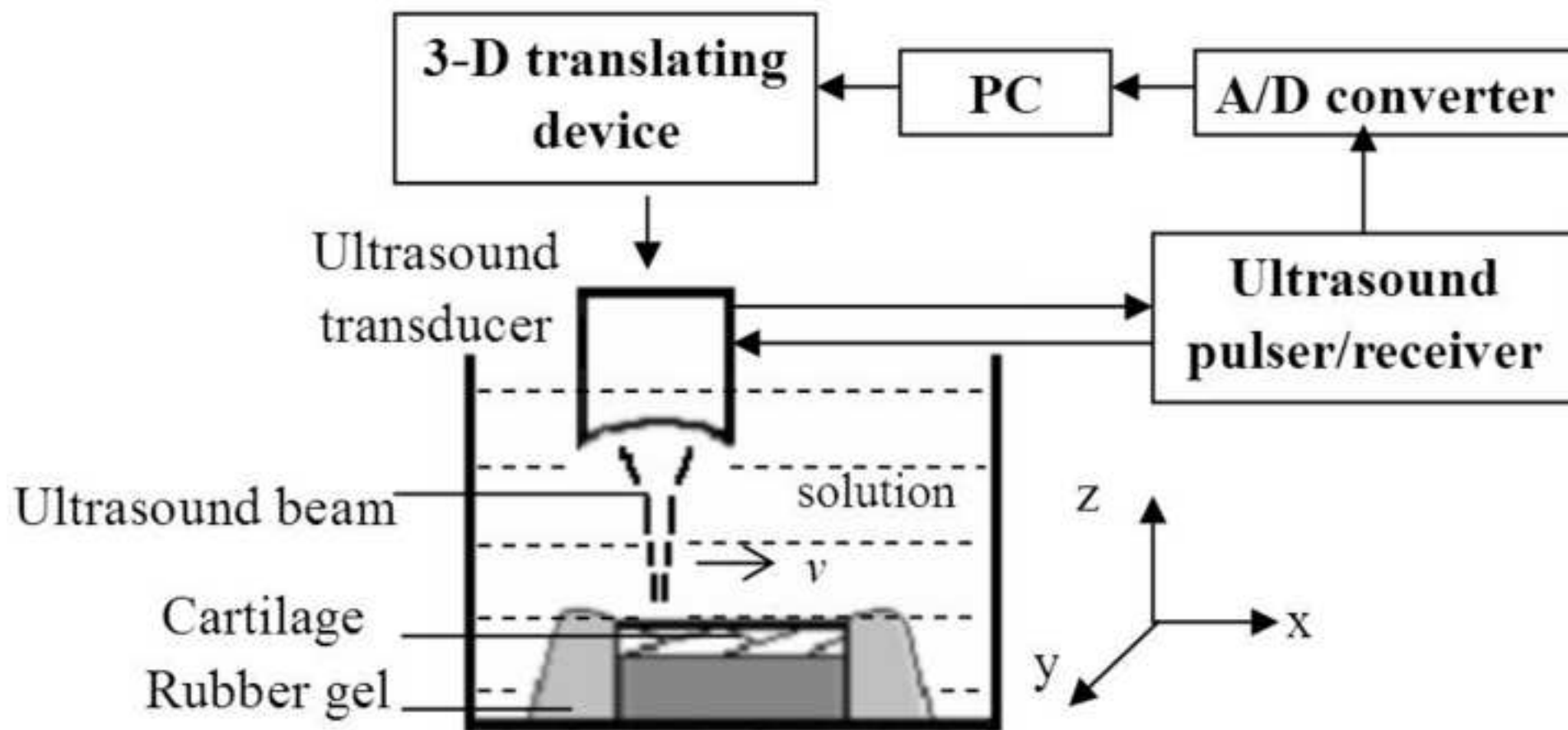


Figure 2b  
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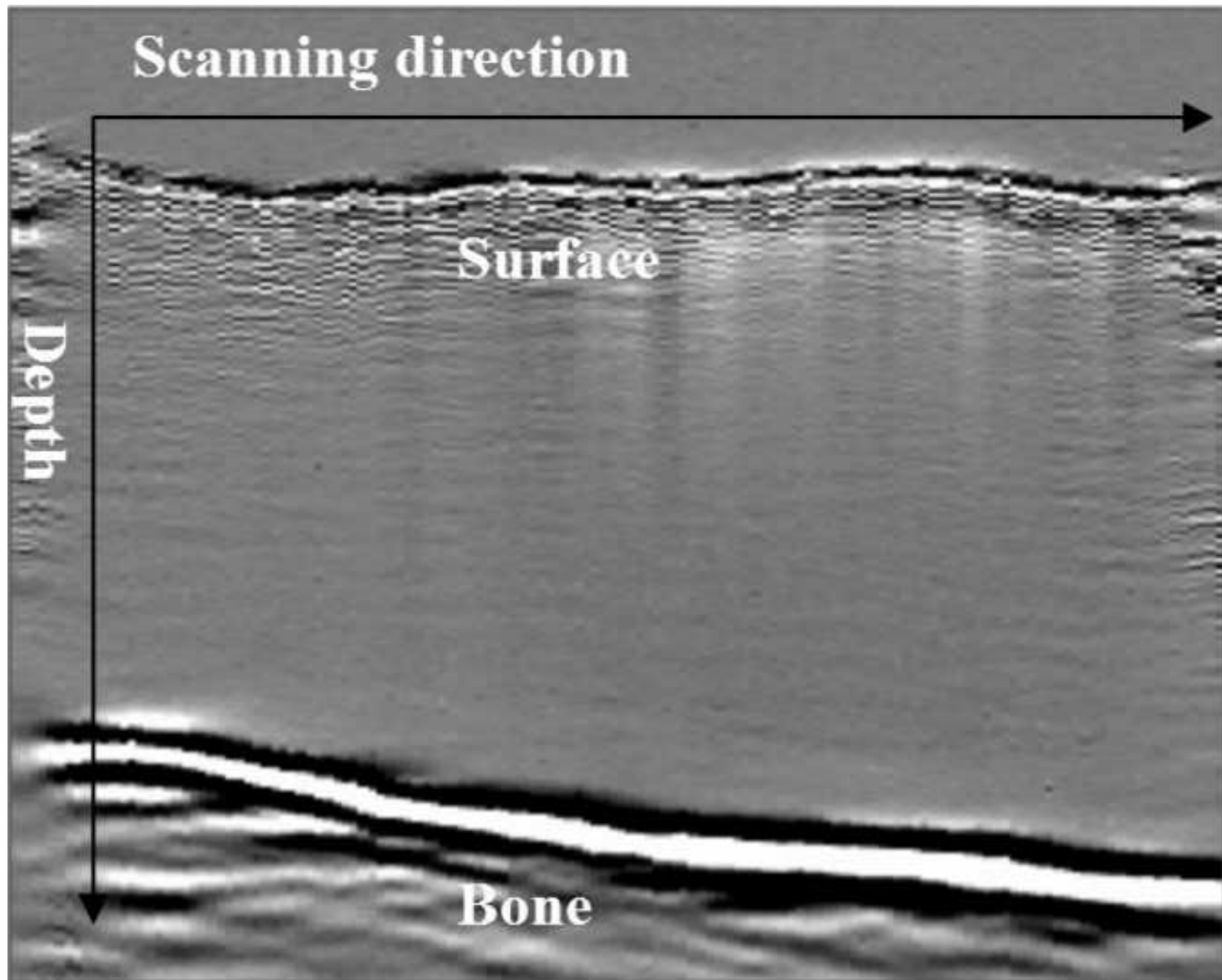
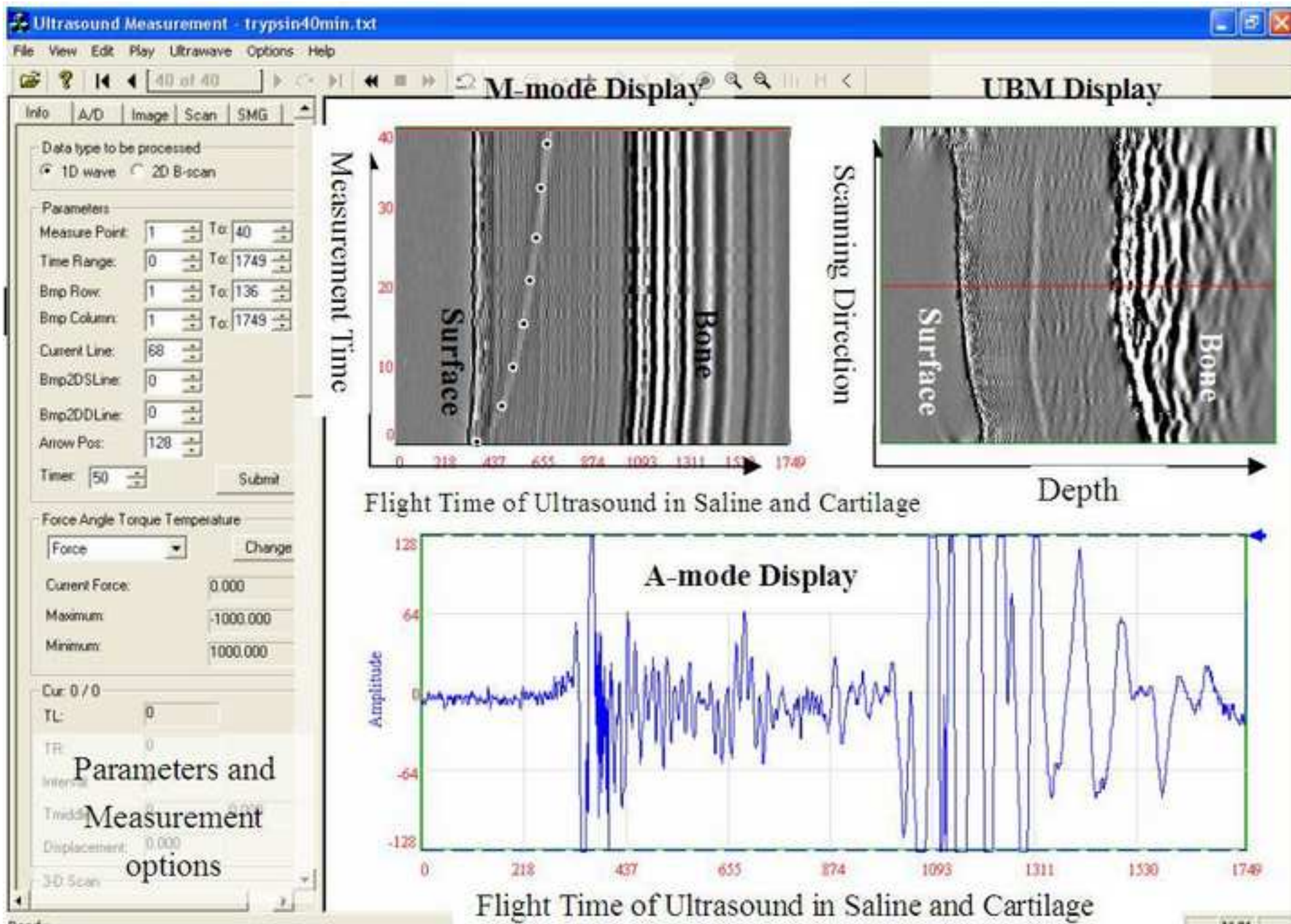
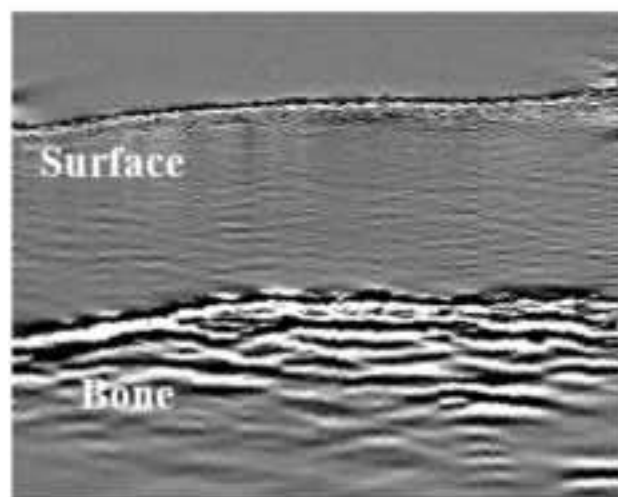


Figure 3  
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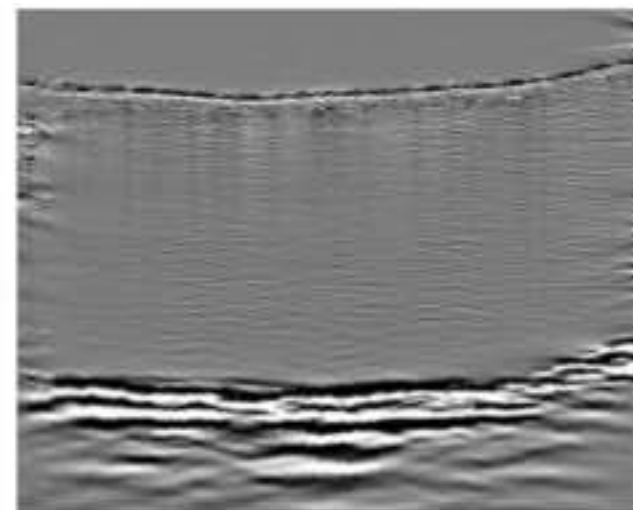




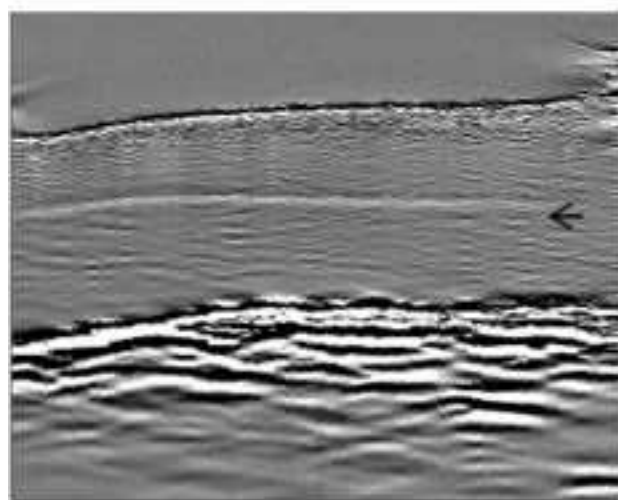
(a)



(e)



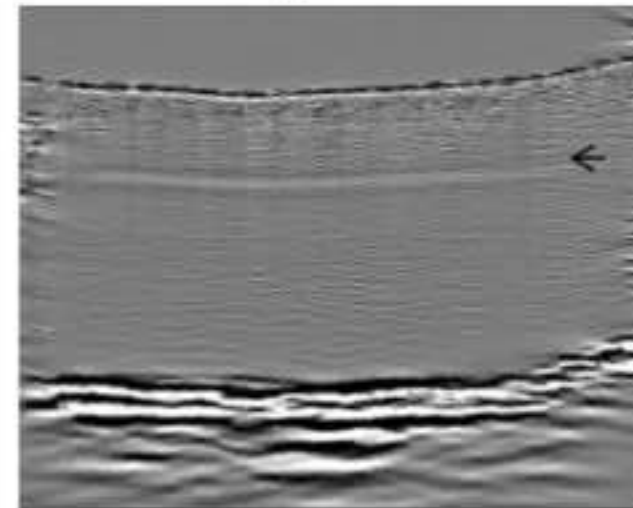
(i)



(b)



(f)



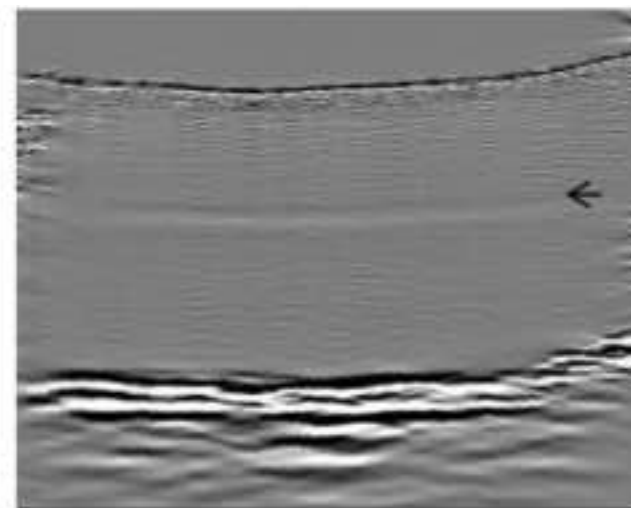
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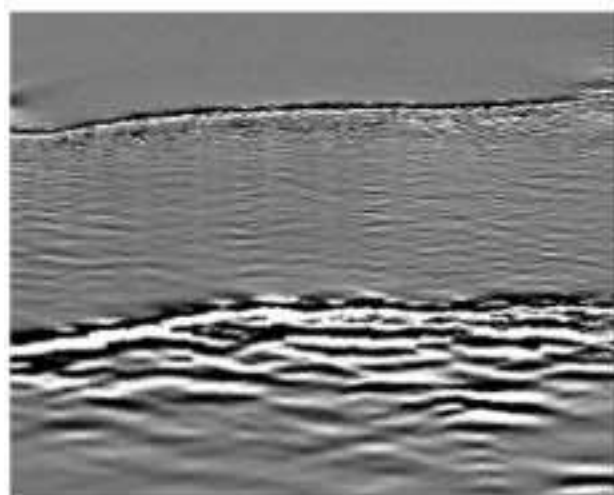
(c)



(g)



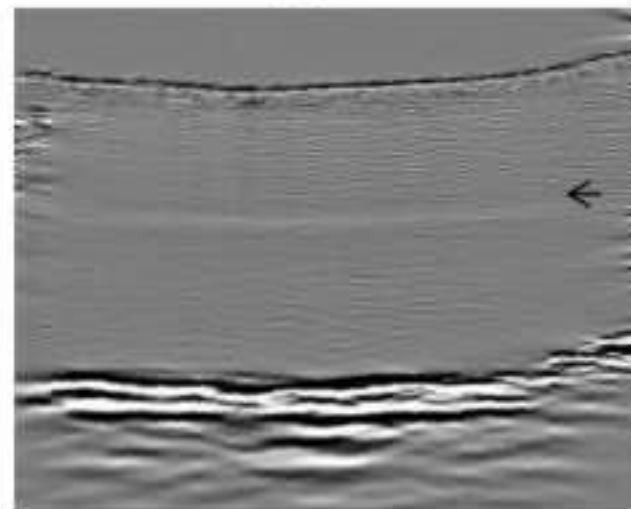
(k)



(d)



(h)



(l)

Figure 5a  
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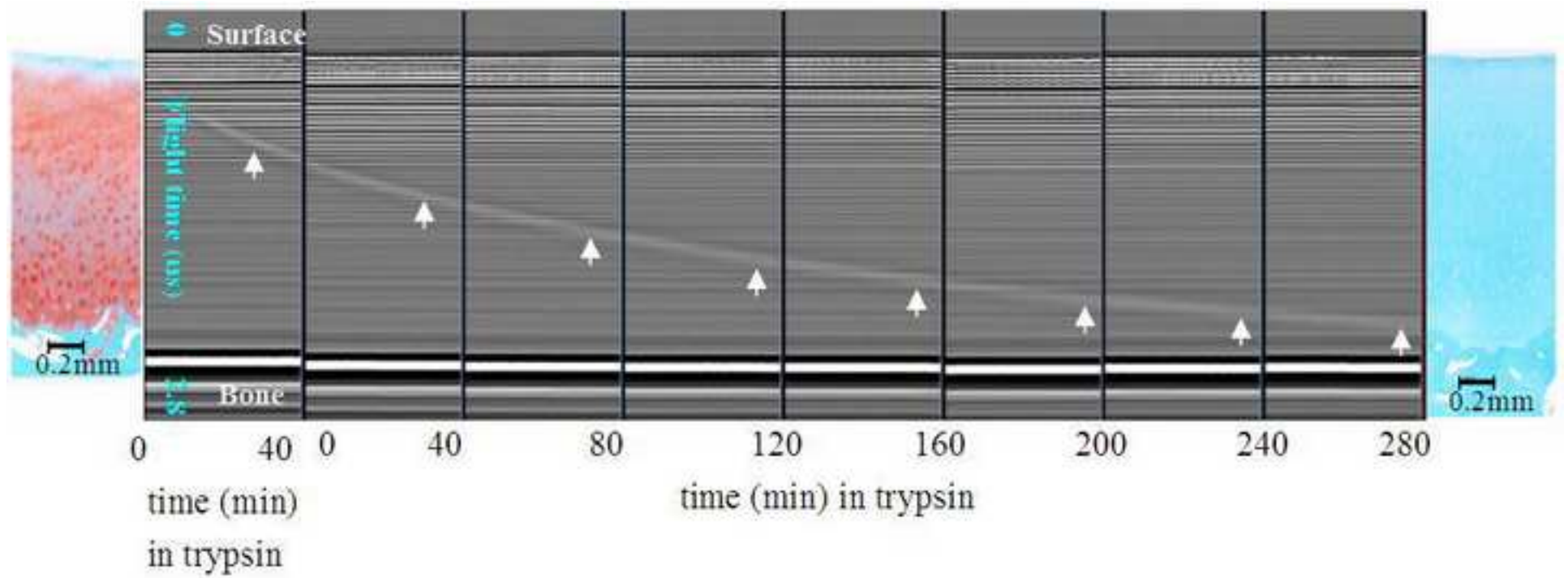


Figure 5b  
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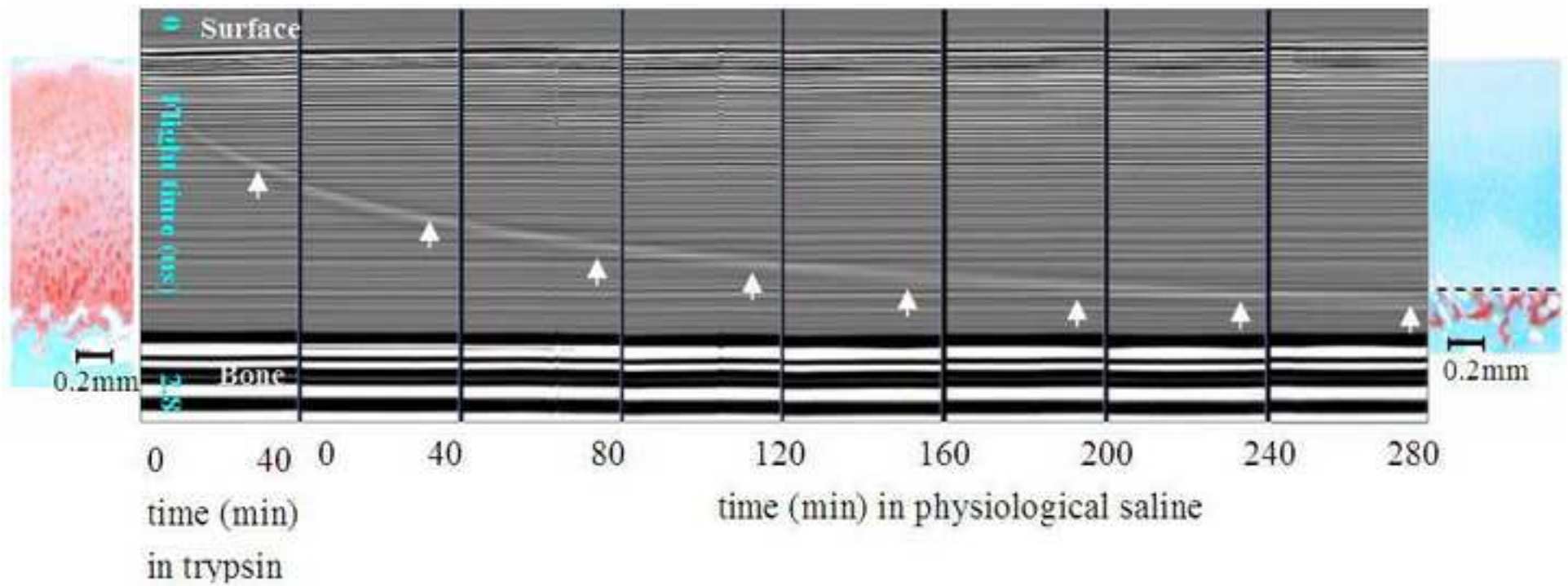


Figure 5c  
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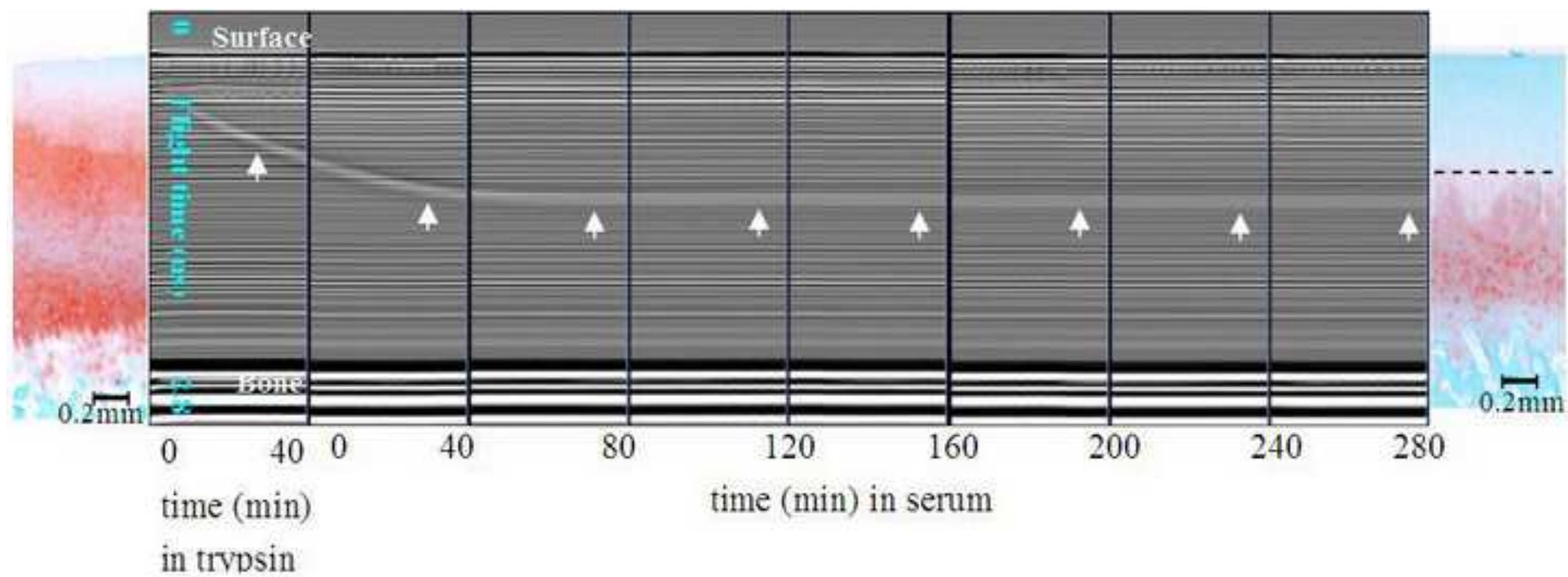


Figure 6  
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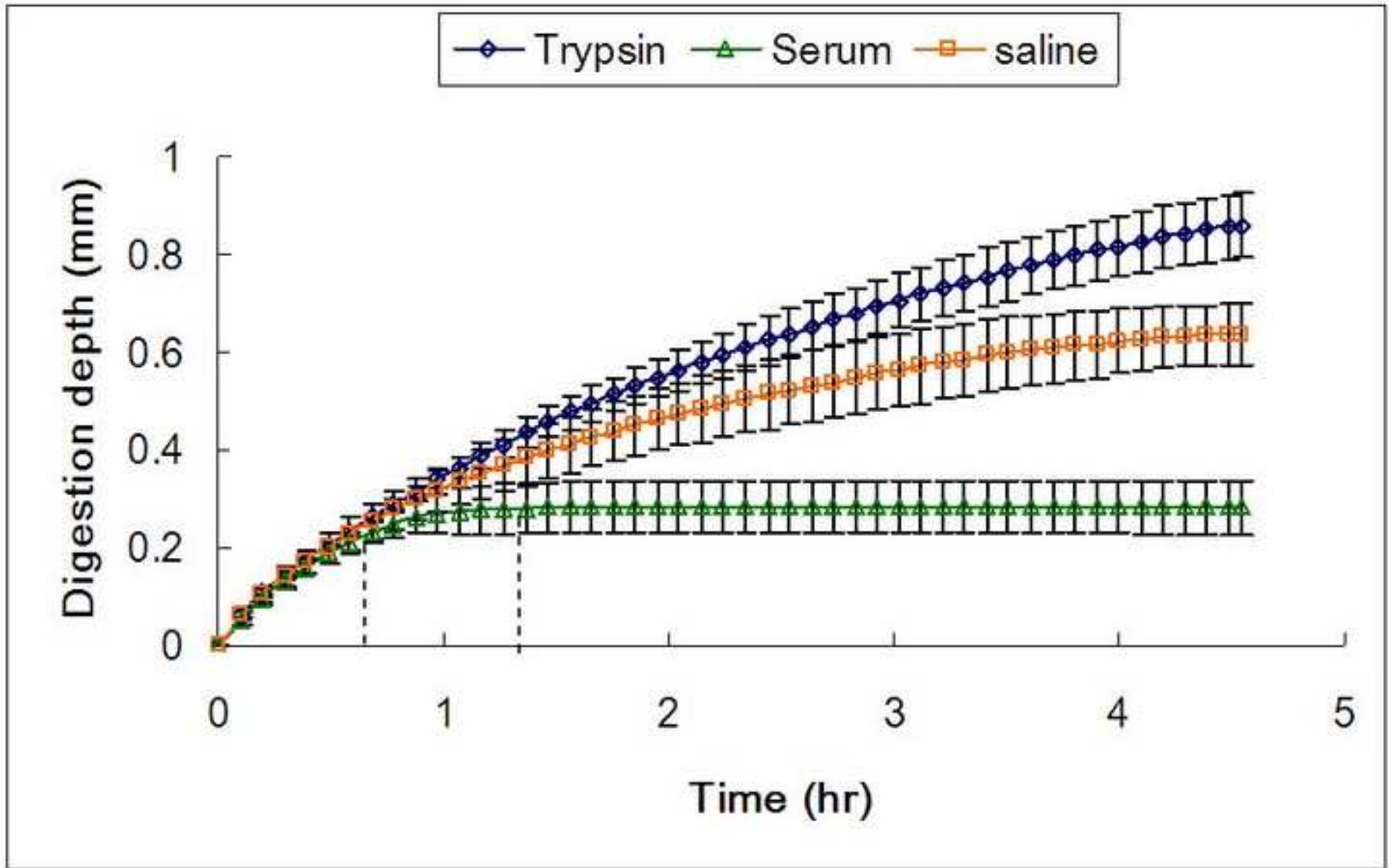


Table 1

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Table 1: The mean digestion fraction of the digested samples measured at the end of 40-min trypsin digestion and 280-min various treatments.

	40min-trypsin (n=9)	4h40min-trypsin (n=3)	4h40min-saline (n=3)	4h40min-serum (n=3)
Ultrasound measurement	0.38±0.08	0.88±0.06	0.83±0.19	0.50±0.02
Histological assessment	-	0.96±0.07	0.84±0.22	0.38±0.06