A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Dynamic response of cells to ultrasound under the variable heights of culture medium in the well

Tsengel Bayarsaikhan

Department of Biomedical Engineering

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

February, 2017

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(Supervised by Professor **Min Joo Choi**)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

February, 2017

This thesis has been examined and approved by

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2016.12

ABSTRACT

In vitro experiments on the ultrasonic effect of cells in culture wells often lack reproducibility and fail successful transfer to other experimental conditions for the similar ultrasonic device settings. It is suspected that the problem is caused by the standing waves formed in the well, although it is not clarified yet. A one dimensional acoustic theory states that the pressure at the bottom of the culture well where cells are attached varies from 0 to twice of the incident wave while the culture medium height varies a half wave length of the incident ultrasound. The present study looked into the dynamic response of the cells in a culture well exposed to ultrasound under the different standing wave conditions controlled by the height of the culture medium. It was observed that the extent of the morphological changes of the cells exposed to ultrasound were significantly different for the different heights of culture medium even if the ultrasonic device output setting remains unchanged. This founding suggests that the standing waves greatly modify the ultrasonic irradiation condition to the cells. The study claims that the parameter such as the culture medium height associated with standing wave effects must be monitored when in vitro ultrasonic cell experiments are performed. Further investigation including the physiological responses of cells is suggested to underpin the present observations.

Key words: low intensity ultrasound, HT-22 cell line, culture well, variable height

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I. INTRODUCTION

1.1 Ultrasound cell stimulation

Ultrasound has potential for the clinical treatment of nerve regeneration in both the central and peripheral nervous system (Ren et al. 2010, Zhang et al. 2009). Ultrasound energy with frequencies above human hearing can be applied to the individual cells to generate the micromechanical strains resulting in the biochemical effects at the cellular level. Non-invasive physical stimulation by ultrasound is known to promote cell viability, proliferation, and change size of the cell body as well as the length of axon and dendrites (or neurite) in neuronal cells. *In vitro* experiments show that modulation of neuronal development has been achieved after the ultrasound exposure during which temporary neurite retraction was triggered and cell body shrinkage was induced (Hu et al. 2013). The neurite retraction and body shrinkage during 10 min ultrasound exposure turned out to initiate the recovery to the neurite lengths over 100 min post-exposure (Hu et al. 2013). In addition, Enhanced neurite elongation was observed in cultured primary rat cortical neurons by 5 min daily ultrasound exposure (Ren et al. 2010).

On the other hand, ultrasonic stimulation induced the alteration of the functions of cultured Schwann cells as demonstrated by promoted cell proliferation (Zhang et al. 2009). In other applications such as collapse of microbubbles ultrasound can temporarily increase the cell membrane permeability (sonoporation) to create a physical route for impermeable agents to enter the cells (Fan et al. 2014).

1.2 Acoustic pressure in culture well

Many *in vitro* studies for ultrasound stimulation effect use the cell lines conventionally cultivated in culture wells. Cells are attached to the bottom of the culture well filled with culture medium during their life time as illustrated in Figure 1.1. In this setup, ultrasound transducer was placed right below the culture well and directly attached to the outer surface of the bottom through which the acoustic energy was delivered to cells sitting on the bottom surface. Several different types of setup are possible

depending on how the ultrasound energy is delivered to the cells of interest as shown in Figure 1.2. Between the different setups, intricate physical development occurs, which makes it difficult to understand on account of the technical differences (Kinoshta and Hynynen 2007). Many researchers prefer to choose the experimental setup of the *culture well on ultrasonic transducer* as shown in Figure 1.2(a) due to its simplicity. However, such *in vitro* studies often lack reproducibility and fail successful transfer to other experimental conditions although ultrasonic environments are similar (Karin et al. 2011).

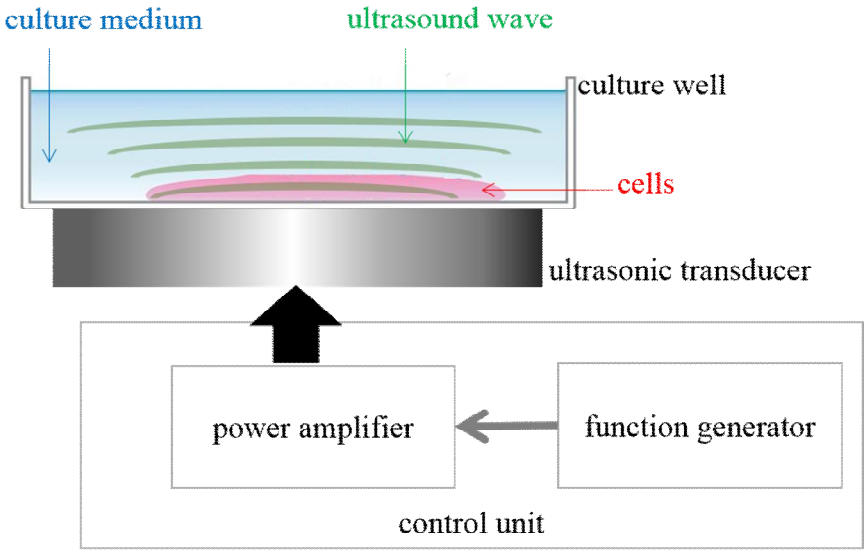


Figure 1.1. An illustration of a typical in vitro experimental setup for the study of cell response to ultrasound

In order to consider ultrasonic technique as a useful tool to stimulate cells, reproducibility of ultrasonic effect should be verified and physical parameters related with acoustic waves and geometry of the setup has to be standardized. Despite the fact that some research groups attempted to optimize the acoustic parameters in their published reports, little attention has been given to the results due to poor understanding of the technical issues of the experimental conditions (Zarnitsyn and Prausnitz 2004, Rahim et al. 2006).

For instance, Schuster et al. (2013) reported that different settings of frequency and dose in an ultrasonic conditions brought different impacts on proliferation, morphology. However, the results of the cell response to the ultrasound are not consistent and little is known about the direct effect of frequency or energy density as a standard protocol. Kinoshita and Hynynen (2007) reported that the reproducibility was enhanced using an absorbing material substituting in medium-air interface. It is suspected that the standing waves formed inside the culture medium can cause differences in results (Hensel et al 2011), which was not clearly clarified yet.

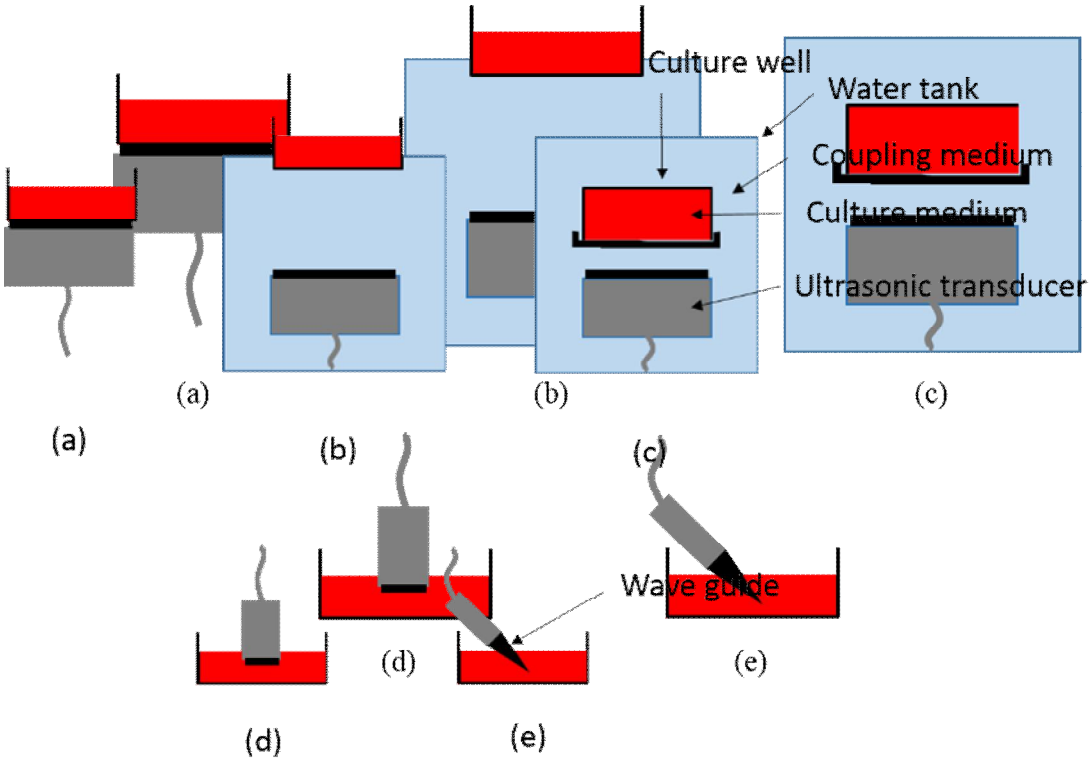


Figure 1.2. Schematic of the various experimental setups for in vitro cultured cell line exposed to ultrasound. (a) Well on ultrasonic transducer, (b) well located in the far-field, (c) sealed and immersed well in the coupling medium of water, (d) and (e) ultrasonic transducer in the culture medium

A simple one dimensional acoustic theory states that the acoustic pressure at the inner bottom of the culture well where cells are attached varies from 0 to its maximum (twice of the incident wave) as the culture medium height varies a quarter wave length of the incident ultrasound (Choi et al. 2012). This

exposed to ultrasound. We hypothesize that the acoustic pressure formed at inner bottom of culture well is subject to the height of medium due to the standing wave effect. Therefore, the amount of ultrasonic-induced stimulation energy can be different depending on the height of medium in the culture well, even if the ultrasonic device setting remains unchanged.

In order to differentiate the dynamic response of the cells to the acoustic pressure, we have monitored the room temperature (RT)-induced cell body shrinkage process. Most cell lines adapted to grow at 37 °C in the incubator can get stresses and changes in their morphology when they are placed at room temperature, usually 20-25°C. As soon as they are exposed to room temperature, they start to respond to the decreased temperature and shrink in their body size as a natural apoptosis process. This RT-induced apoptosis process can be fostered by applying an extracellular physical stimulation such as the acoustic pressure.

In this study, we have investigated the acceleration of the RT-induced apoptosis process by delivering the acoustic power to the cells at room temperature. Instead of monitoring and measuring the ultrasound response of the cells inside the incubator as most other experiments have been performed, we installed the measurement setup in the ambient temperature intentionally. In addition to the acceleration of the apoptosis process, we have differentiated the standing wave effect on the dynamic response of the cells exposed to ultrasound by altering the height of the culture medium in the well. Therefore, depending on the strength of the acoustic stimulation on the cells, we expect to observe the difference in their morphological changes.

II. MATERIALS AND METHODS

2.1 Cell culture

HT-22, an immortalized hippocampal neuronal cell line, was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and incubated at 37℃ humidified air with 5% CO_2. Cell density was 3×10^4 cells/well in a culture well with 35 mm in diameter (Figure 2.5-2).

2.2 Measurement of ultrasound intensity

The experimental setup of acoustic power measurement was shown in Figure 2.2. A single element, ultrasonic transducer (V302, Panametrics NDT, USA) with flat surface was used as the source of ultrasound (Figure 2.2 (a)). The transducer has the aperture diameter of 25.4 mm and is resonated at the frequency of 1 MHz. RF signal is supplied by a function generator (33250A, Agilent Tech., USA) to generate continuous sine wave and is amplified in a power amplifier (75A250, Amplifier Research Inc., USA).

Ultrasound power meter (UPM-DT-1AV, Ohmic Ltd., USA) was used to measure total acoustic output power of ultrasonic beam in watt [W] (Figure 2.2 (b)). The principle of the measurement is based on the radiant force from the ultrasonic field. A positioning clamp is used to hold the transducer in de-gassed water above a conical target. The ultrasonic energy passes through the water to reflect off the target and is then absorbed by the rubber lining. The radiant power is directly proportional to the total downward force on the target. This weight is then transferred through the target support assembly to the electro-mechanical load cell inside the scale (Preston 1991). The spatial average temporal average intensity of ultrasound can be calculated as total acoustic power divided by the target area (Acevedo and Das-Gupota 2002) (Figure 2.2 (c)).

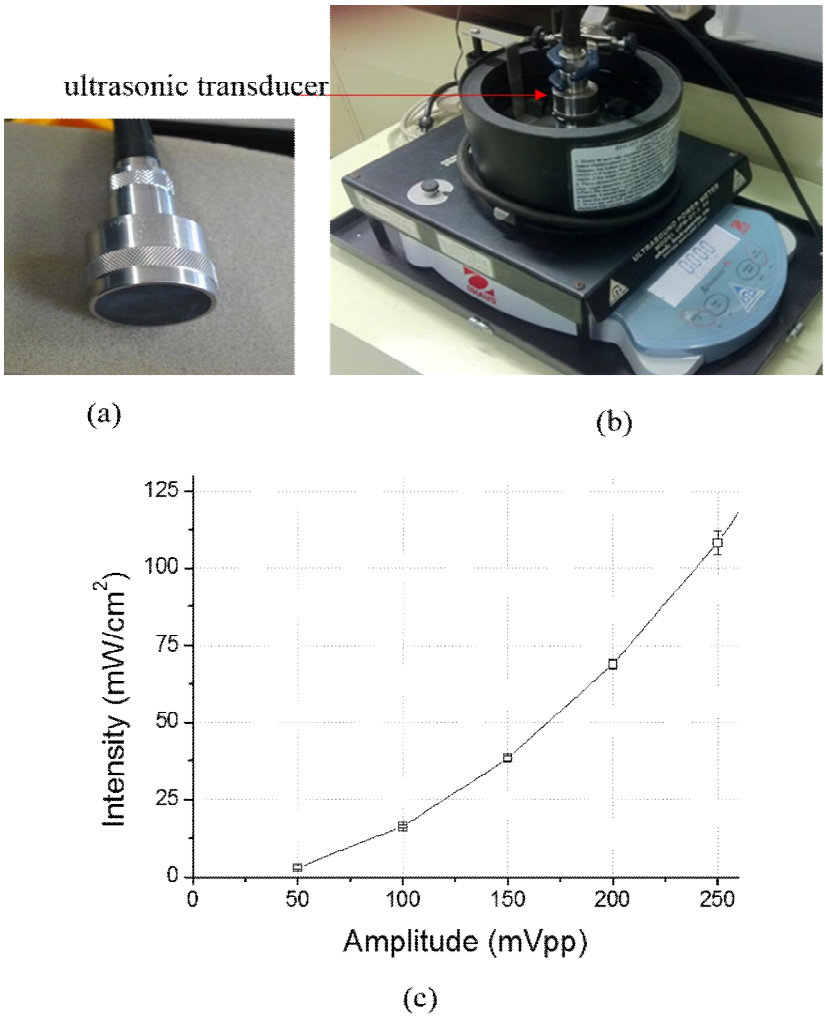


Figure 2.2. Measurement of ultrasonic power. (a) A single element, ultrasonic transducer (V302, Panametrics NDT, USA) with flat surface, (b) the ultrasound power meter (UPM-DT-1AV, Ohmic Ltd., USA) and (c) ultrasound intensity.

2.3 Pressure estimation at the bottom of the culture well

The standing wave formed in the culture medium of the well was predicted using plane wave propagation theory. Assuming the water is used as the culture medium, pressure at inner bottom of culture well is given by Kinsler et al. (1999)

$$p(z=0,t) = p_0[1 + R(-2ik_cH)]\exp(i\omega t) \quad \text{..... (1)}$$

$$R = \frac{Z_{air} - Z_{water}}{Z_{air} + Z_{water}}$$

$$\omega = 2\pi f_0$$

$$k_c = \omega / c$$

Here, Z_{air} and Z_{water} are acoustic impedance of air and water, respectively. R is the reflection coefficient, ω is the angular frequency, f_0 is the incident ultrasound frequency, c is the sound speed of water, k_c is the wavenumber in water, H is the height of water, and p_0 is the initial pressure.

The predicted peak amplitude of the standing wave varies with the culture medium height which repeats by a half wavelength height. Figure 2.3 displays the peak pressure against the culture medium height. As seen in Figure 2.3, the pressure magnitude varies from zero (node) to the twice of incident wave (antinode), being repeated by every half wavelength height. The present study considered the four different heights matched to the four pressures equally divided from zero to the maximum value (marked by A, B, C, and D where the A represents the antinode and the B is node). The ratios of pressure at each height are 2, 1.5, 0.5 and 0 to incident pressure respectively.

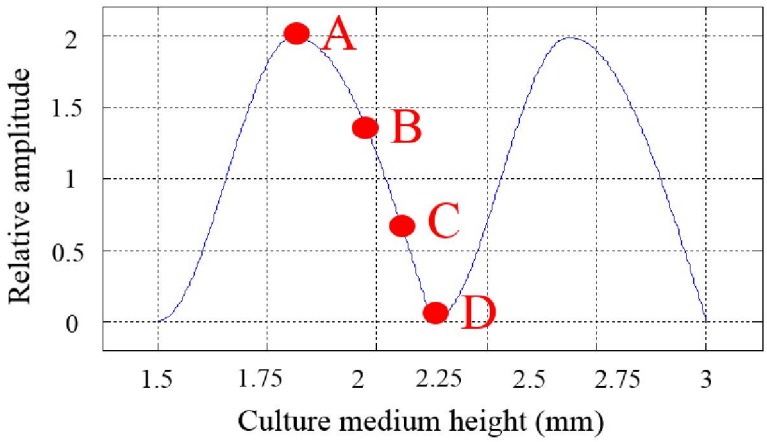


Figure 2.3. The predicted peak amplitude of the standing wave against the culture medium height: the four different heights matched to the four pressures equally divided from zero to the maximum value, marked by A, B, C, and D where the A represents the antinode and the D is node.

2.4 Height control of culture medium

Under the conditions that the ultrasonic frequency is 1 MHz and the culture medium is assumed to be acoustically equivalent to water, the values of the four heights used in the present experiment are A=1.875mm, B=2mm, C=2.125mm, and D=2.25mm, as illustrated in Figure 2.4. The height of the culture medium in the well was measured from the ultrasonic pulse reflected at the medium-air interface. Figure 2.4(b) shows the ultrasonic pulses measured for the four heights (A, B, C, and D) using a 10 MHz ultrasonic transducer (V311, Panametrics NDT, USA) driven by a Pulser/Receiver (PR5800, Panametrics NDT, USA), which were acquired on a digital oscilloscope (LC574AL, 1GSps, LeCroy, USA) (Figure 2.4(a)). The time of flights of ultrasound between the inner bottom of culture well and the medium-air interface were respectively 2.5, 2.667, 2.832 and 3μs for the four different heights of A, B, C, and D (Table 2.1).

Table 2.1 Time of flight (TOF) of ultrasound between the inner bottom of culture well and medium-air interface for the four different culture medium heights of A, B, C, and D defined in Figure 2.3 ~Figure 2.4.

	TOF (μs)	**Height (mm)**
A	**2.500**	**1.875**
B	**2.667**	**2.000**
C	**2.832**	**2.125**
D	**3.000**	**2.250**

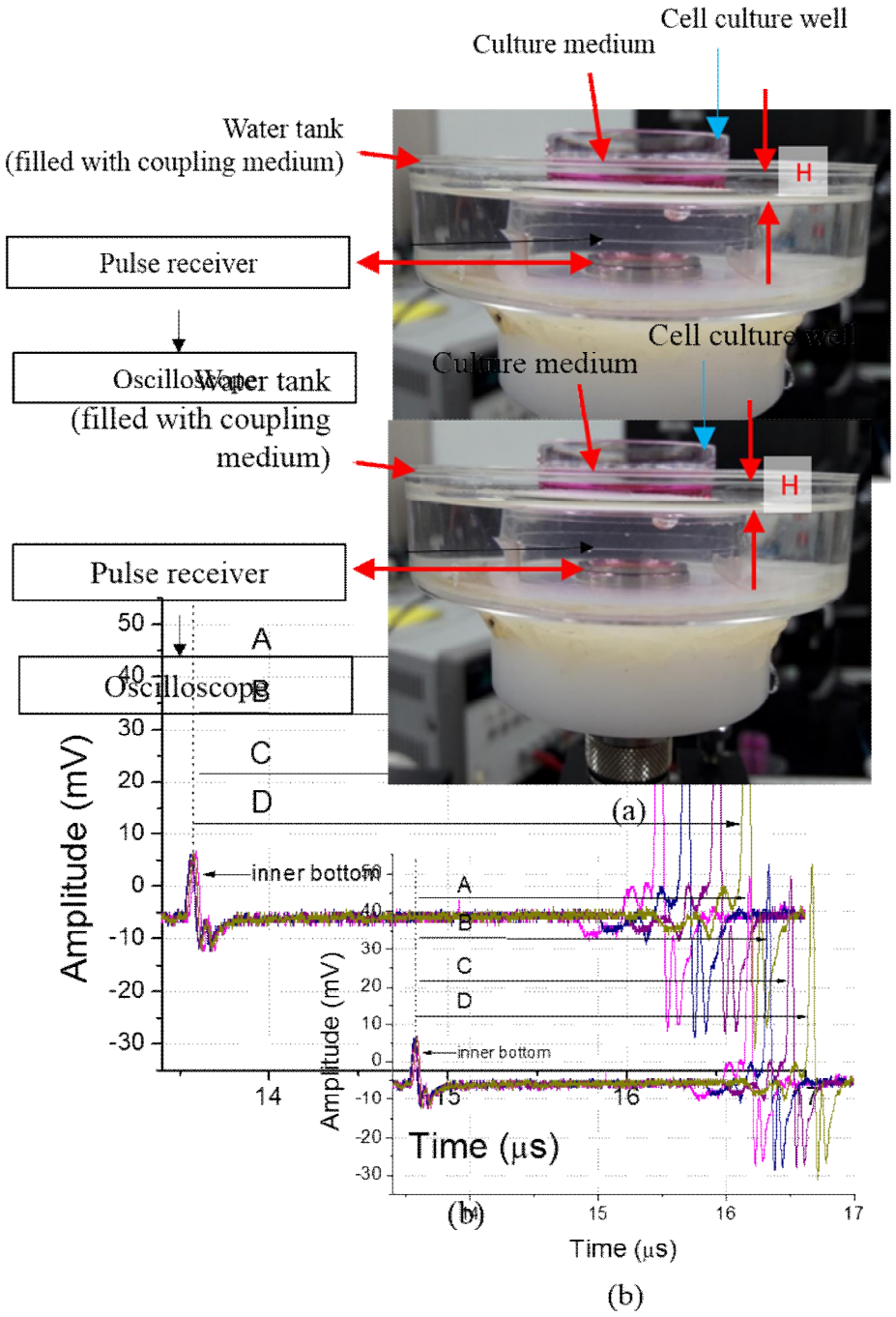


Figure 2.4 Ultrasonic measurement of the height of the culture medium in the well: (a) experimental setup with a 10 MHz ultrasonic transducer and (b) ultrasonic pulses reflected at the medium-air interface for the four heights (A, B, C, and D). The time of flight of ultrasound for the four different culture medium heights are provided in Table 2.1

2.5 Microscopic imaging

The HT-22 cell line was imaged by a digital camera (EOS 5D Mark III, Canon Inc., Japan) adapted with a microscope (CH-30, Olympus Inc., Japan) carrying an objective lens (10x, Olympus, Japan) (Figure 2.5-1). One of the eye pieces was replaced by the digital camera and the other was used for the light illumination whose current was controlled by a current source to regulate the light intensity. The culture well with neuron cells was mounted on the ultrasound transducer holder that was inserted on the sample stage of the microscope. The cells in the culture well were illuminated through the objective for the reflection mode operation which was enhanced by a reflector located right below the bottom surface of the culture well. The reflector was obtained from a smart phone backlight reflector that has over 99% reflectivity and small attenuation of the acoustic power. In order to reduce the temperature increase from the energy absorption of the medium by the ultrasound wave, a homemade fan circulates the air around the sample stage. The medium temperature was monitored by the Thermal camera (T630sc, FLIR Inc., Sweden) as shown in Figure 2.5-1(a). The acquired image has the size of 5760 x 3840 pixels and the spatial resolution of 0.227 μm/pixel.

A typical image of the HT22 cell obtained using the present microscope (equipped with the ultrasonic transducer under the culture well) is shown in Figure 2.5-2(b), contrasting it to the image (Figure 2.5-2 (a)) obtained using a conventional microscope. As seen in Figure 2.5-2, the image with the present microscope is virtually characteristically the same as that of the conventional microscope. This confirms the present microscope can be used for the real time morphological observation of the cell under ultrasonic irradiation. For the analysis purpose, the contrast of cell images was further enhanced by Contrast-Limited Adaptive Histogram Equalization (CLAHE) in Matlab software.

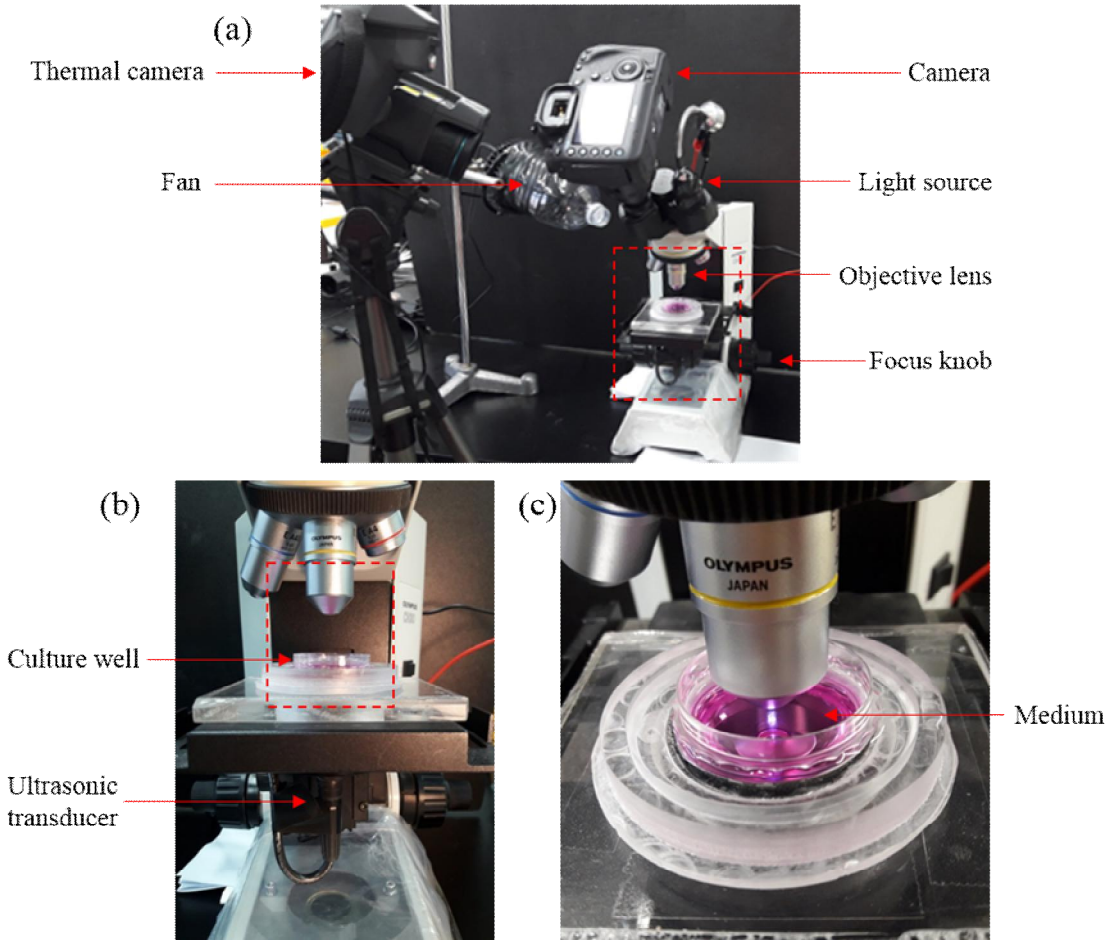


Figure 2.5-1. The present experimental setup: (a) photograph, (b) culture well (top view) and transducer, (c) culture medium.

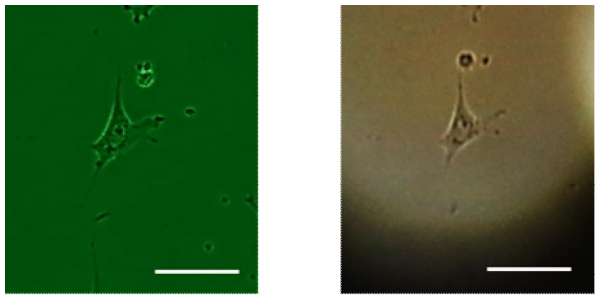


Figure 2.5-2 A typical image of the HT-22 cell, (a) taken from a conventional microscope and (b) obtained with the present microscopic setup. (Scale bar = 50 μm)

2.6 Ultrasonic exposure condition

A 1 MHz ultrasonic transducer (V302, Olympus, USA) used to irradiate the ultrasound wave to the cells, driven by the electrical signal which was generated by a function generator (33250A, Agilent tech., USA) and amplified by a power amplifier (75A250, Amplifier Research, USA). The transducer surface was contact through a coupling medium to the outer bottom of the culture well. Ultrasound was transmitted from 25.4 mm diameter ultrasonic transducer surface area to the bottom of the 35 mm culture well via water (Figure 2.6). The HT-22 cells were exposed to ultrasound, after 24 hours seeding, with a nominal intensity (I_{TASA}) of 50mW/cm^2 and operated at the continuous wave mode for 10 and 15 minutes for length measurement and circular cell count, respectively. The ultrasonic device setting was chosen from the measurement of the ultrasonic power meter (UPM-DT-1AV, Ohmic Ltd., USA) to produce the ultrasound power of 50mW/cm^2 (Figure 2.2). It should be noted that the measured nominal intensity is the expected one from the power meter calibration, which should vary in reality depending on the height of the medium.

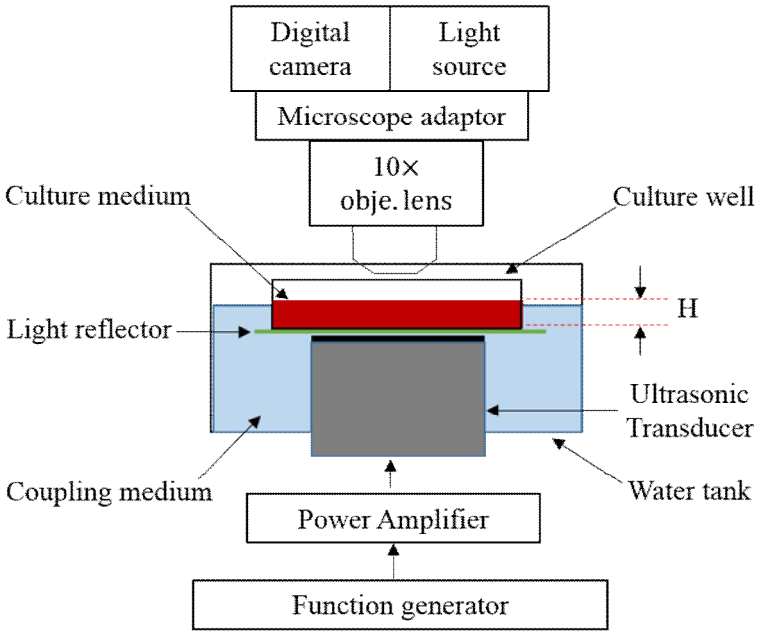


Figure 2.6. The schematic of the present experimental setup for ultrasonic irradiation to the cells cultured in the well, together with the microscopic monitoring device.

2.7 Analysis of ultrasound-induced morphology changes

The microscopic images of the cells at each experimental condition were processed to look into any morphological changes such as cell body shrinkage and displacement, neurite retraction and expansion and area differences. Image shows cell body, axon, dendrite (or neurite) and nucleus of cell body inside. Ultrasound irradiation generates small ripples on the surface of the medium and we had to refocus the image to compensate the disturbance due to the ultrasound. The identification of the cell boundary was made by manually using automatic selection tool named 'Magnetic lasso tool' in image processing software with default settings 'width 10, contrast 10, frequency 57' (Photoshop CC, Adobe Inc., USA) due to its poor quality of image contrast. Subsequent image processing procedures to quantify the extent of the morphological response of the cell, thresholding, binary conversion etc, were performed using MATLAB (R2015a, MathWorks Inc., USA). Temporal changes in the area of the cell boundary including neurite, relative to that at the starting time of ultrasonic irradiation, were calculated to view the extent of the morphological response of the cells to ultrasound.

III. RESULT

3.1 The morphology of HT-22 cells

When the cells were grown for 24 hours after seeding, we took the cell image at room temperature using our customized imaging system with the ultrasound transducer installed. The typical HT-22 cells characterized by cell body or soma, nucleus, dendrite and axon or neurite were illustrated in Figure 3.1. Cell body contains the nucleus and neurites are extend from the cell body and attached to a substrate. One of the most common cell types is the one whose body and neuritis are fully expanded as shown in Figure 3.1 (a), which looks healthy and stable. Figure 3.1 (b) shows a cell body had an axon. HT-22 cells eventually become circular in their shape as they are exposed to the room temperature for longer time as shown in Figure 3.1 (c) and we believe this is the cell death stage (apoptosis) where they stop the biological functioning that the loss of cell volume or shrinkage, nuclear condensation, internucleosomal DNA fragmentation, and apoptotic body formation (Bortner et al. 2002 and Shimaru et al. 1997). Cells then detached from the bottom surface of the well and float around in the medium.

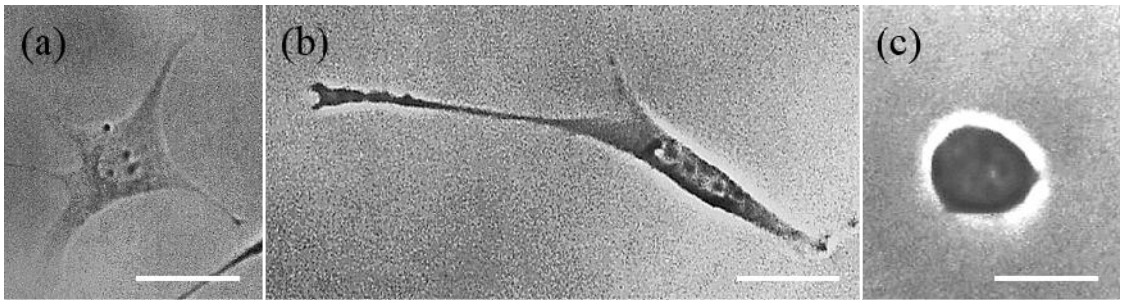


Figure 3.1. Typical image of the HT-22 cell line, taken from the present microscope system. (a) Cell body and neurite were extended, (b) cell body had an axon and (c) cell body had no neurite (circular cell). (Scale bar 20μm).

3.2 Ultrasound induced cell responses

The cells that were transported from the incubator to the ultrasound transducer installed imaging system were imaged every 20 second for 15 minutes while ultrasound pressure was applied. Figure 3.2 shows an example of the morphology changes of HT-22 cell during ultrasound irradiations. In this figure, we selected two typical cell responses with time, the neurite retraction and the dynamic activities of cell body, for different medium heights, 1.875, 2.0, 2.125, 2.25 mm and control (1.875mm). The neurite retraction is one of the most noticeable variations of the cell morphology due to the environment change and the physical stimulation like the ultrasound pressure. The neuritis tend to retract rather rapidly in less than 2 minutes in most of the cases once they start to do so (Figure 3.2 (a)). In addition, cell body becomes circular as soon as the neurites are finished retraction. Some cells with circular body shape show different responses in this experiment such as neurite expansion, active alteration inside the body as shown in (b)'s of Figure 3.2. From this figure, we have concluded that the length measurement of the cell and the circular cell count could be two good measures to demonstrate the morphology responses to the ultrasound pressure as we will show in the following sections (section 3.5 and 3.6). We have used these two parameters to illustrate the morphological responses to the ultrasound pressure quantitatively because it is not easy to tell the differences among the results from different medium heights.

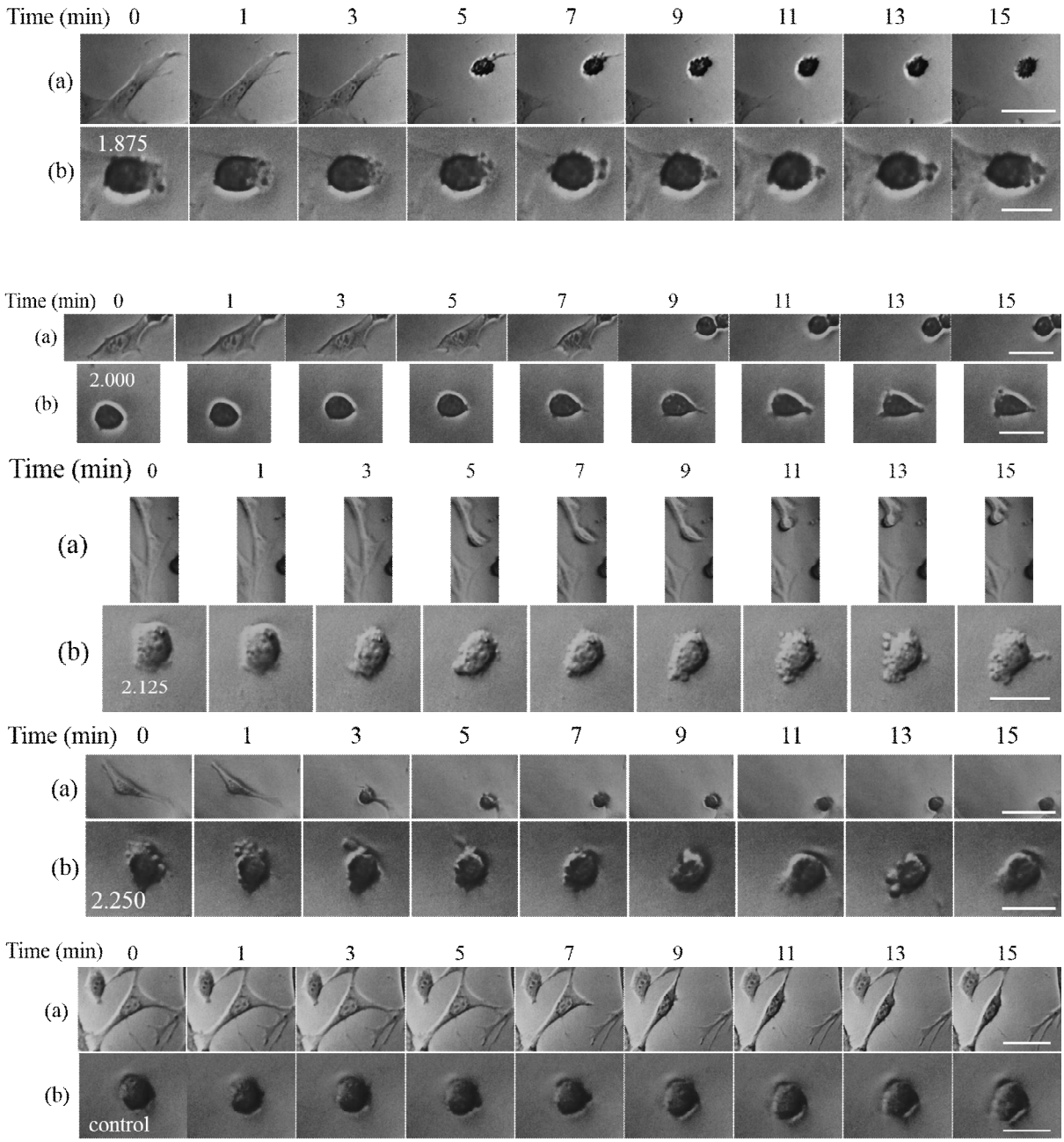


Figure 3.2. The morphology changes of HT-22 cell during ultrasound irradiations. (a) Cell body and neurite retraction, (b) cell body response by ultrasound exposure for different medium heights, 1.875, 2.0, 2.125, 2.25 mm, and control (1.875mm). (Scale bar 20μm).

3.3 Dynamic changes of cell body and neurite

Figure 3.3 shows the variations of neurites and cell bodies during ultrasound irradiation. Cell in Figure 3.3-1(a) shows the generation of a new neurite next to the other one after 9 minute of ultrasound irradiation and then retracted at 14 minute. More distinct generation of a neurite was shown at 8 minute in Figure 3.3-1 (b) where the body was expanded as well. In the case 2.125 mm (figure 3.3-1 (c)), cell neurite was elongated at 10 minute. However cells doesn't show any growth in neuritis in Figure 3.3-1 (d) and (e) which are for the medium height of 2.25 mm and control(1.875 mm), respectively, both of which are due to the low acoustic pressure.

Figure 3.3-2 shows Dynamic morphological changes of the cells to the ultrasonic irradiation were further clarified by the afterimages constructed by overlapping all of the cell images (captured every 1 minute for the time period of ultrasonic irradiation) to one image and averaging it in colors. In the afterimages for the cases B and C, Figure 3.3-2 (b) and (c) shows the cell movement and changes in the body greater than other cases D and E, resulting in larger variations in color. In the case of A, the largest acoustic pressure was applied to the cell but it didn't show larger variation of the body whereas the sub-cellular activities inside the body was observable in Figure 3.3-1(a). From these images, it is speculated that the neurites expanded as well as the cells moved during the ultrasound irradiation.

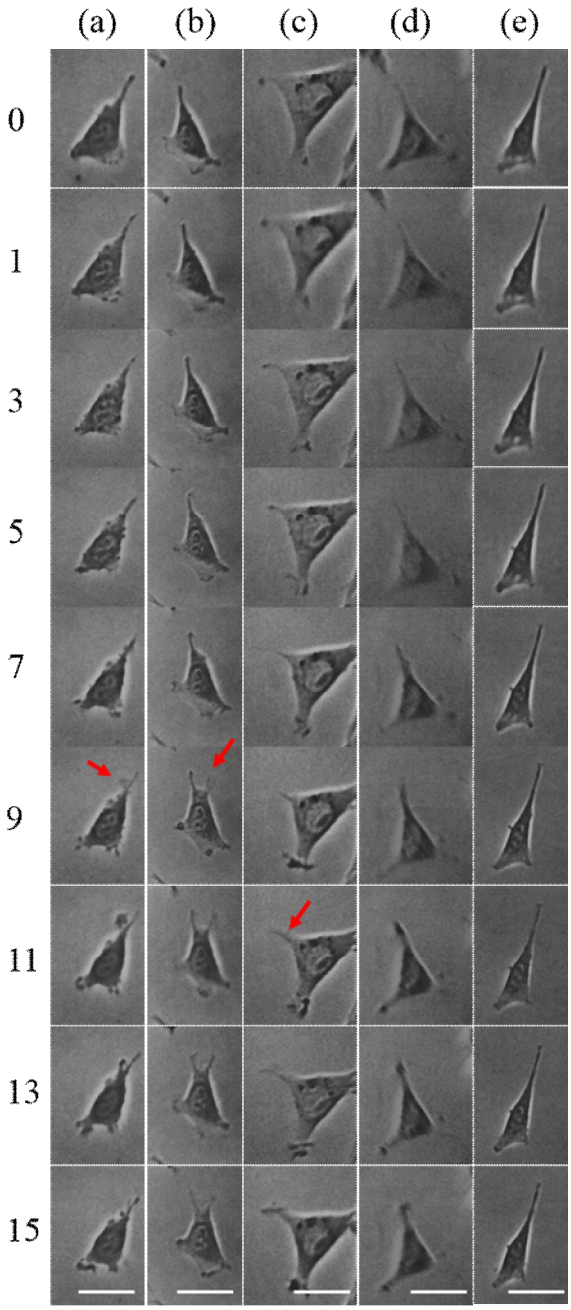


Figure 3.3-1. The variations of neurites and cell bodies during ultrasound irradiation. (a) A, (b) B, (c) C, (d) D, (e) control. (a) Cell body and neurite were moved actively and expanded at 9 min, (b) cell neurite was expanded at 8 min, (c) cell neurite was moved and elongated at 10 min, and cells were inactive in (d) and (e) (scale bar 50μm).

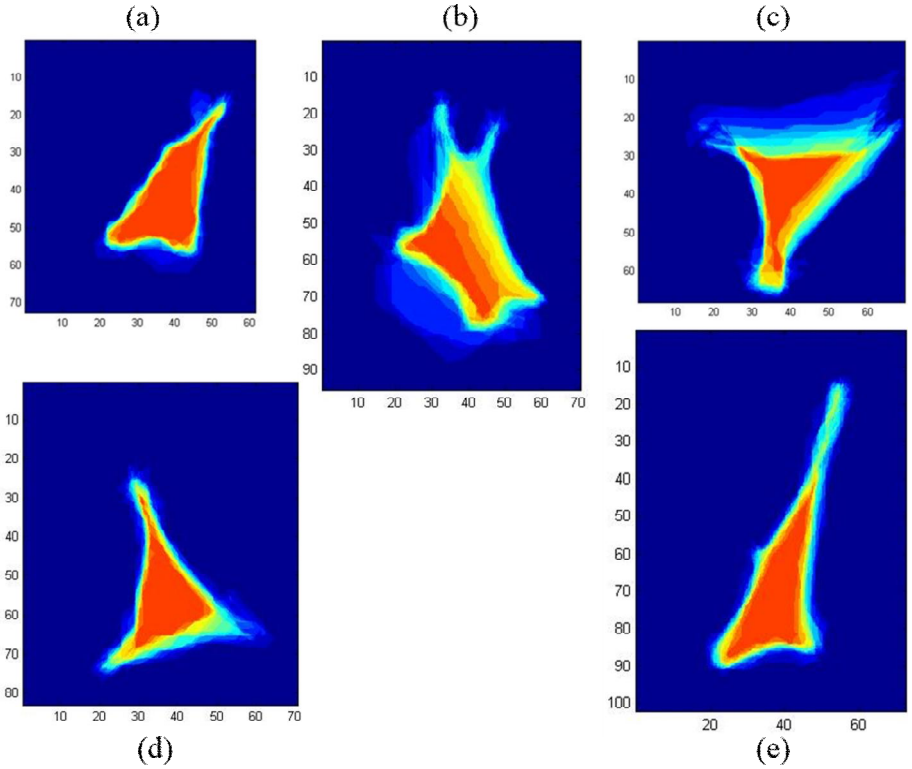


Figure 3.3-2. Afterimages of the cell morphological changes for the 5 cases of the 4 different culture medium heights and the control, constructed by overlapping all the images of the cell (captured every 20 second for the time period of ultrasonic irradiation) to one image and averaging the image in colors.(a) A, b) B, (c) C, (d) D, (e) control

3.4 Length changes

Cell body shrinkage is the most conspicuous cell response in the process of cell apoptosis (Martin et al. 2005 and Kristiansen et al. 2014). Healthy cells are triggered to change in their morphology when they are exposed to uncomfortable environment starting with the neurite retraction followed by the body shrinkage (Shimura et al. 1997 and Shimura et al. 1998). Figure 3.4-1 shows the temporal variations of the cell morphology for 15 minutes after less than 5 minutes of preparation procedure for different medium heights with ultrasound exposure and the control without ultrasound. In the case of A(1.875 mm medium height), the elongated cell with a neurite start to retract in the body and the neurite after 5 minutes from the start of the ultrasound exposure. After 11 minutes, the cell morphology didn't change much and became a circular cell indicating the cell death stage. The cell with three neurites in case B(2.0 mm) retracted one of the neurites and altered the body shape after 5 minutes. Interestingly the body shape varied from circular to oval shape between 5 and 15 minutes. In the cases of C(2.125 mm) and D(2.25 mm), the length start to decrease after 5 minutes. The control (1.875 mm) cell with no ultrasound irradiation start to reduce the length of the neurite and area of the cell body after 9 minutes where the triggering time is delayed compared to the cases with ultrasound exposure.

Figure 3.4-2 shows the temporal variations (plotted in the vertical direction) of the cells acquired under the 4 different heights of the culture medium ((a), (b), (c) and (d)) and no ultrasonic irradiation ((e) as a control) (plotted in the horizontal direction). Figure 3.4-2 (a) shows the length of cells for different medium heights and control and the normalized ratio of the length changes is redrawn in Figure 3.4-2 (b) for the comparison. The cases with ultrasound exposure show the decrease in the length started around 4 minutes whereas the control cell length start to reduce around 8 minute. From these results, we could conclude that the ultrasound exposure triggered the retraction of cell length earlier than the control case with no ultrasound exposure.

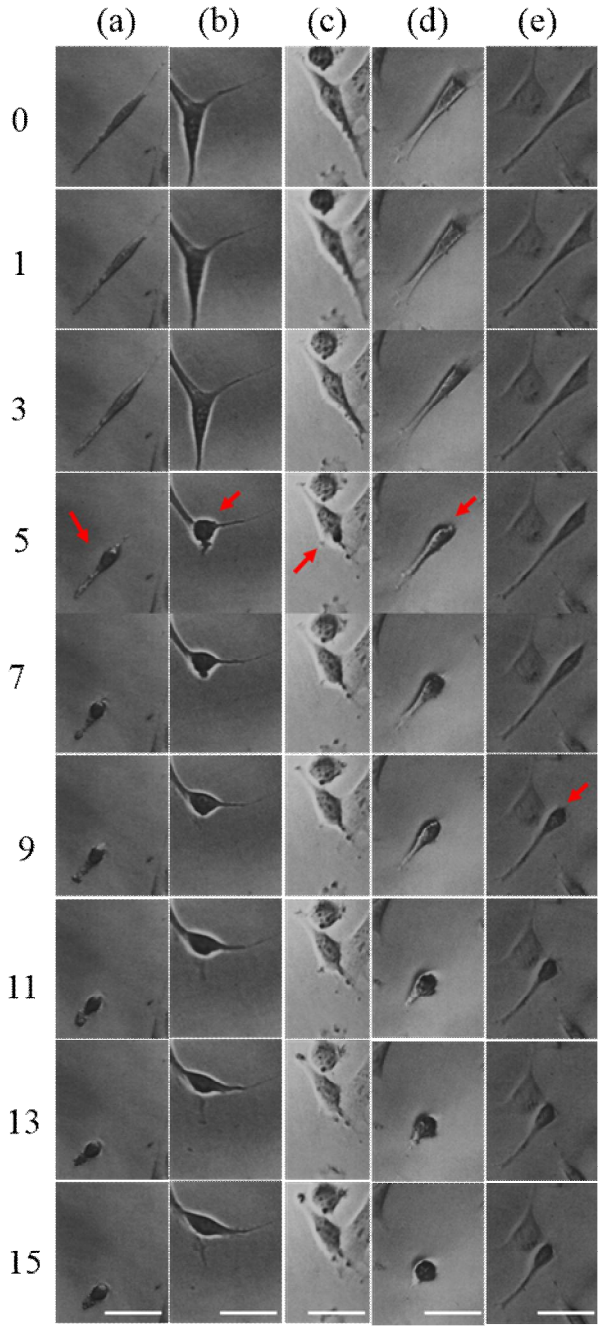


Figure 3.4-1. Temporal variations of the cell morphology. (a) A, (b) B, (c) C, (d) D, (e) control. (a) Cell neurite retracted at 5 min, (b) cell neurite retracted at 5 min, (c) cell neurite retracted at 5 min, (d) cell neurite retracted at 5 min, (e) cell body was changed to smaller at 9 min, but neurite didn't retract. (Scale bar 50μm)

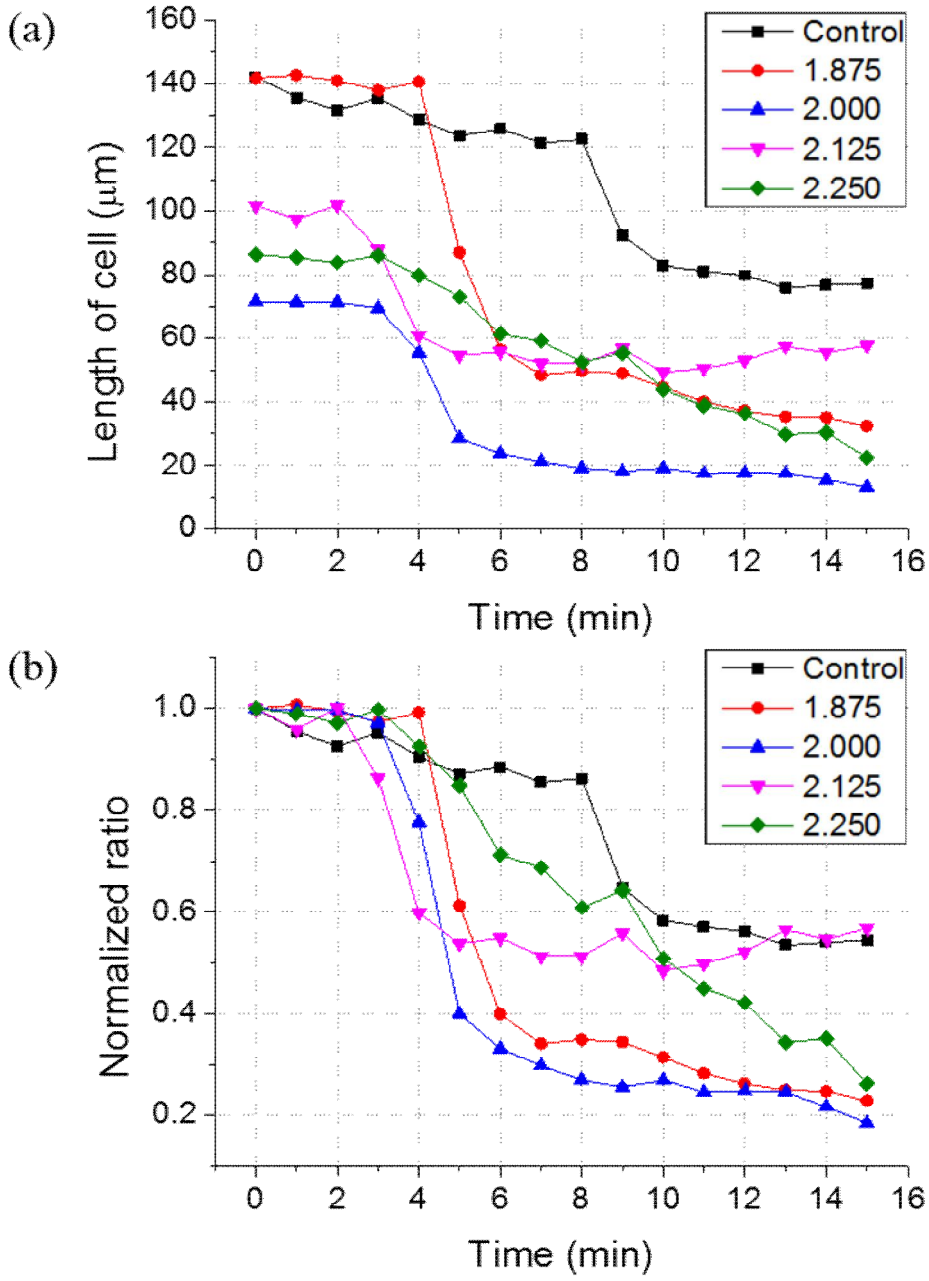


Figure 3.4-2. The temporal variations of the cells. (a) Shows the length of cells for different medium heights and control. (b) The normalized ratio of the length changes

3.5 Length changes after 5 minute at room temperature

Quantitative analysis of cell response to the ultrasound wave was performed by monitoring the length of the cells as a function of time. We have analyzed the cell images for the time period when the cells were exposed to the ultrasound irradiation to monitor the real time cell activities. Figure 3.5(a) shows the averaged length variation of the 20 cells for 4 different heights of the medium and a control well. Most cells for 10 minutes have gradually been decreased in their length due to the temperature-induced apoptosis. However, as we expected, cells that were experienced the ultrasound pressure have retracted more during this period compared to the control cells. The red line representing the medium height of 1.875 mm shows a larger retraction in their length after 5 minutes of ultrasound exposure compared to the cells of the control. The height of 2.0 mm (blue line) also shows a slightly faster retraction of the length after 7 minutes of exposure as illustrated in Figure 3.5 (a). The other two heights of 2.125, 2.25 mm show similar retraction trends compared to the control case.

The medium height of 1.875 mm made the cells retract to about 66% of their initial length and 2.0 mm height gave rise to 74% of retraction of their initial lengths. On the contrary, the cells for 2.125, 2.25 mm and the control were retracted to about 81%, 82%, and 85%, respectively, as shown in Figure 3.5 (b). This implies that the height of 1.875 mm gives the largest ultrasound power, about twice of the incident wave, to the cells due to the fact that the cells are sitting at the anti-node point of the standing wave. The height of 2.25 mm generates the node point at the bottom of the well where the net acoustic power is to be close to zero, which results in the similar response to one from the control well.

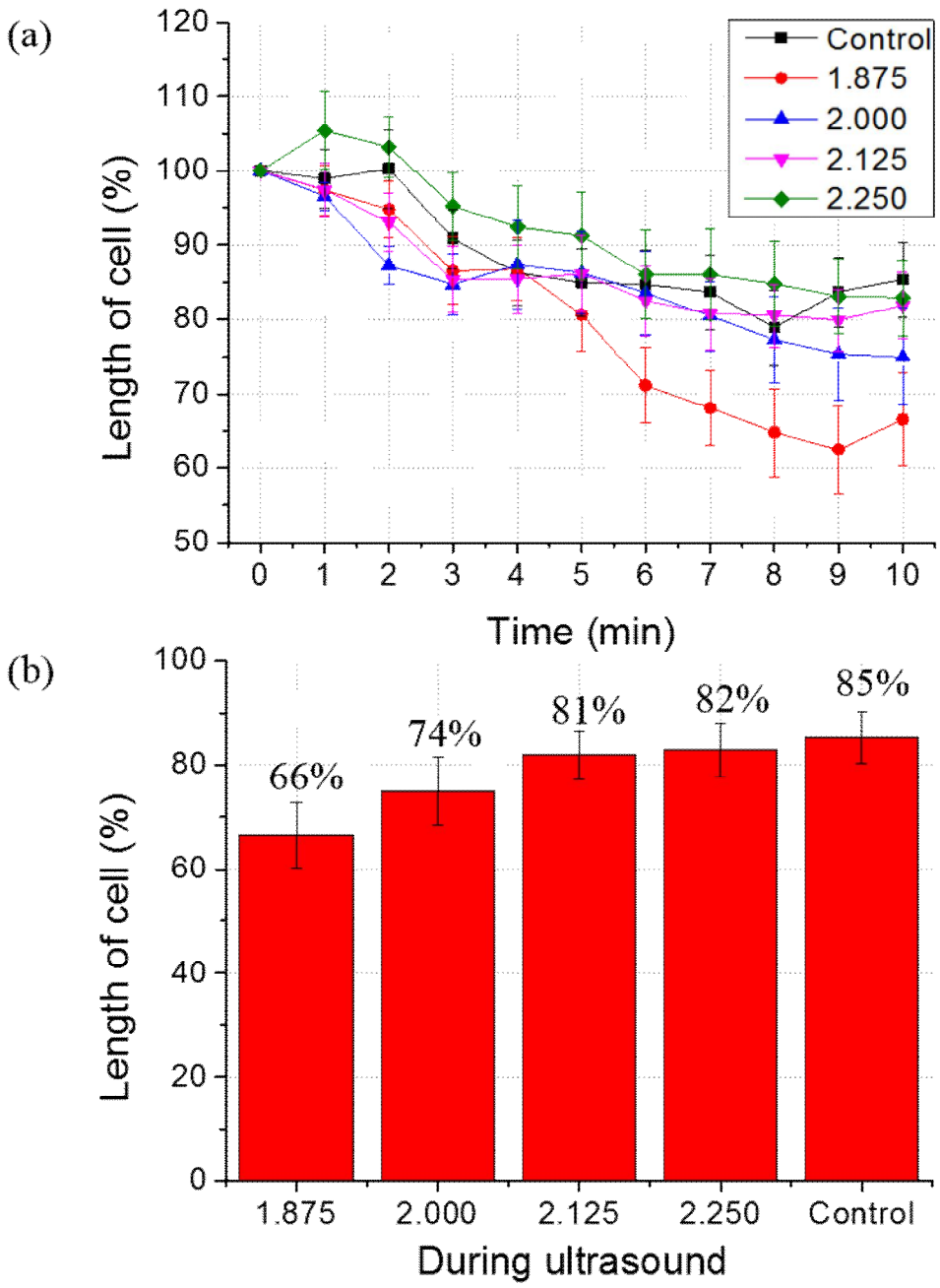


Figure 3.5. Lengths retraction of cells for 10 minute ultrasound exposure after 5 minutes of wait in room temperature. (a) Normalized cell length in percentage with time, (b) Bar graph of the normalized length of cells for different medium height conditions

3.6 Circular cells count

Another indicator that shows the temporal response of cells to the ultrasound is counting the number of circular cells as a function of time. We counted the circular cells, i.e. intuitively dead cells, from the beginning of the ultrasound exposure to the end of it by every minute as shown in Figure 3.6-1. There were already a countable number of circular cells on the bottom of the culture well even when the cells were taken out from the incubator due to the natural and random process of apoptosis as illustrated with the dotted red circles in Figure 3.6-1(a). Being exposed to the ultrasound irradiation, cells shrink in their size and lead to death becoming a circular shape, which results in an additional increase of the number of circular cells which is indicated in Figure 3.6-1(b) with the dotted white circles.

Figure 3.6-2, 3 shows the number of circular cells normalized by the total number of cells and then by the first value at 0 minute as a function of every minute for 4 different height of the medium. All 4 different results were compared respectively to the result of the control that has the same height but no ultrasound irradiation. The number of circular cells for the smallest height (1.875mm) of the medium that corresponds to the maximum acoustic power to the cells was increased faster compared to the control as shown in Figure 3.6-2 (a). The height of 2.0 mm that gives the second largest power resulted in a faster increase in the number of circular cells compared to the control as shown in Figure 3.6-3 (b). However, Figure 3.6-3 (c) and (d) shows that the height of 2.125 and 2.25mm makes no big difference in the rate of increase in the number of circular cells compared to the control. In the case of 2.25mm height, the number of circular cells for the ultrasound exposure was saturated to the total number of cells in the culture well. From Figure 3.6-2, 3, it could be concluded that cells responded to the ultrasound pressure by a faster increase in the number of circular cells depending on the height of the medium.

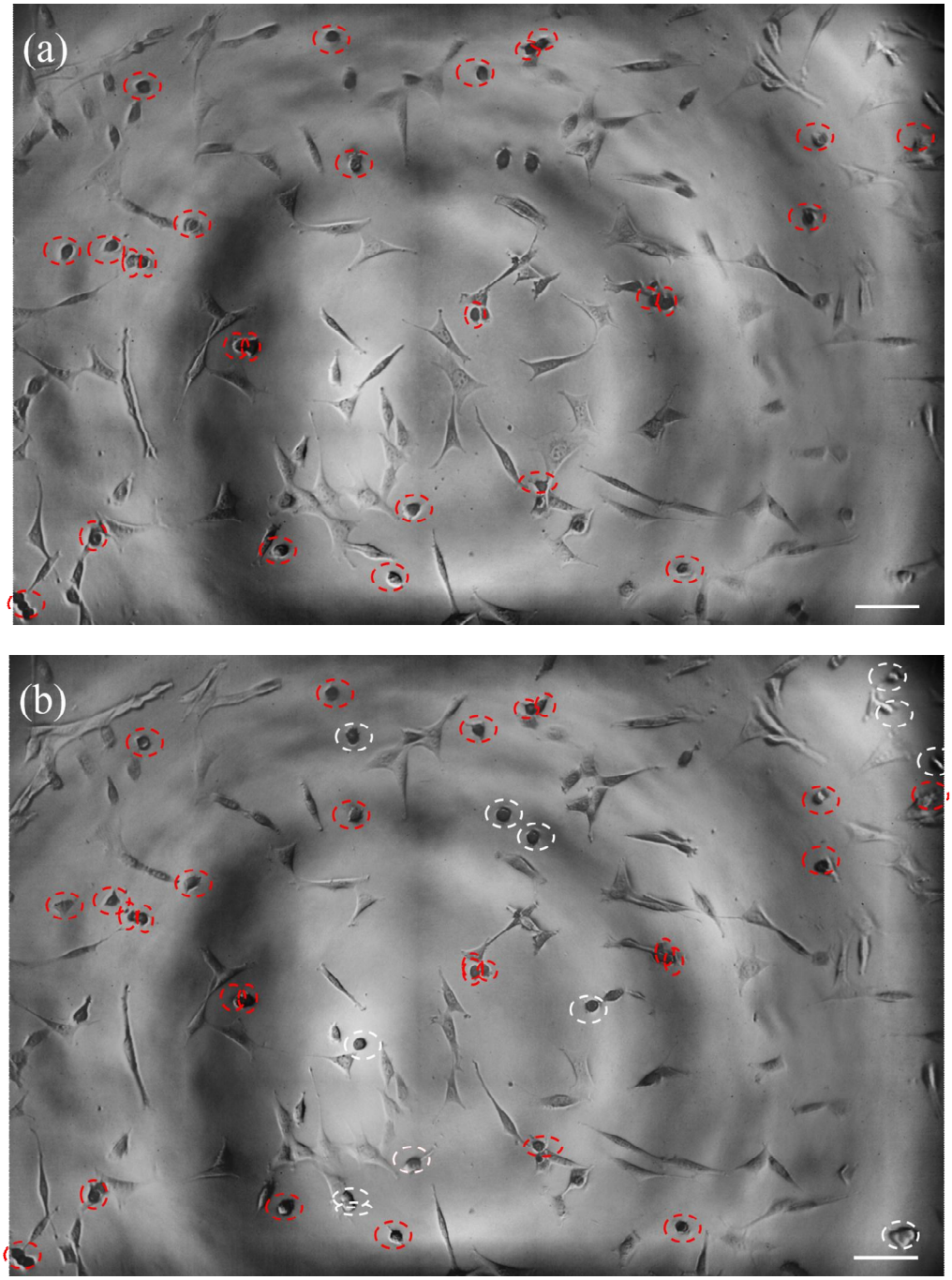


Figure 3.6-1. Temporal response of cells to the ultrasound. (a) Circular cells inside of red round beginning ultrasound exposure, (b) circular cells inside of white round end of ultrasound. (Scale bar 100μm)

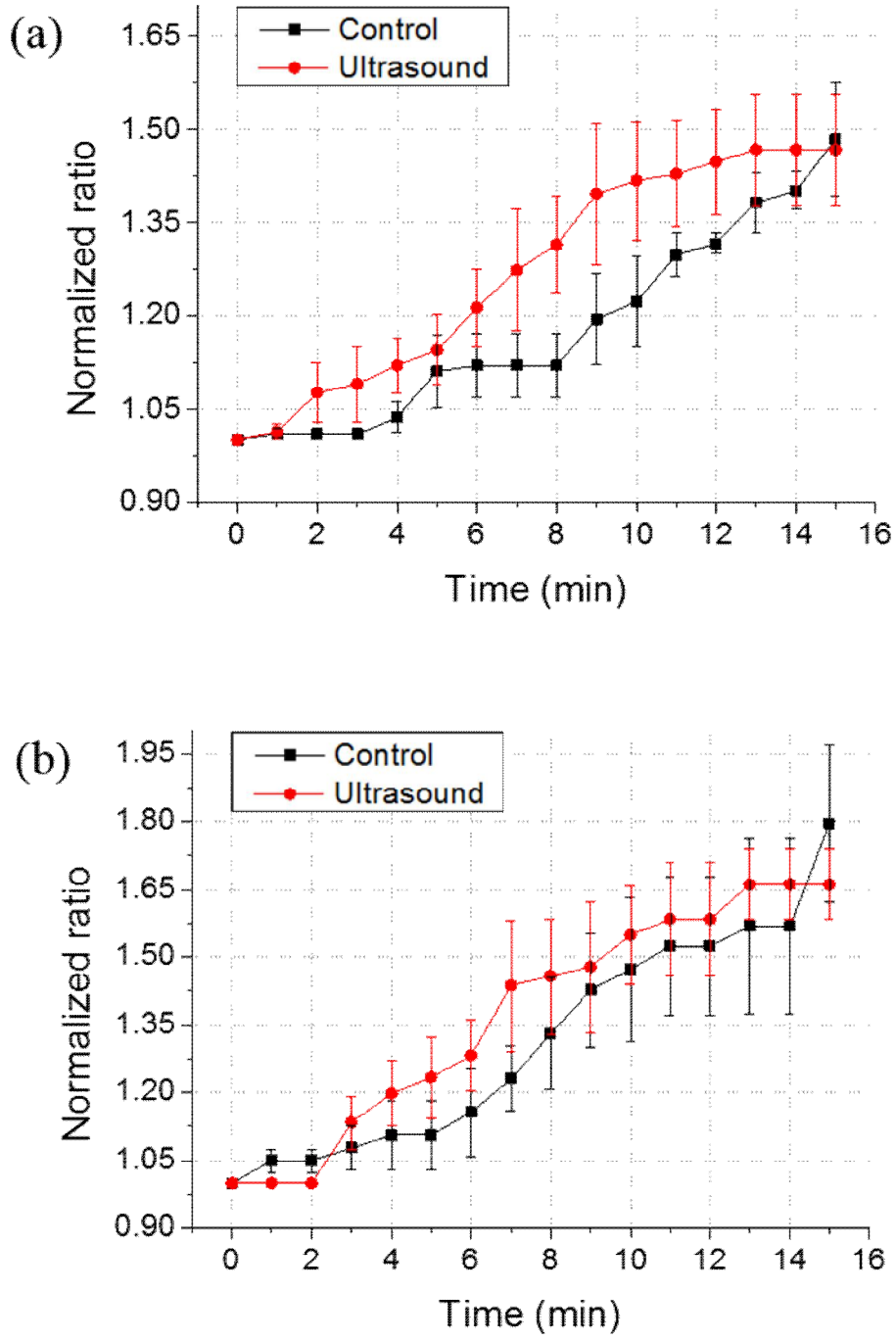
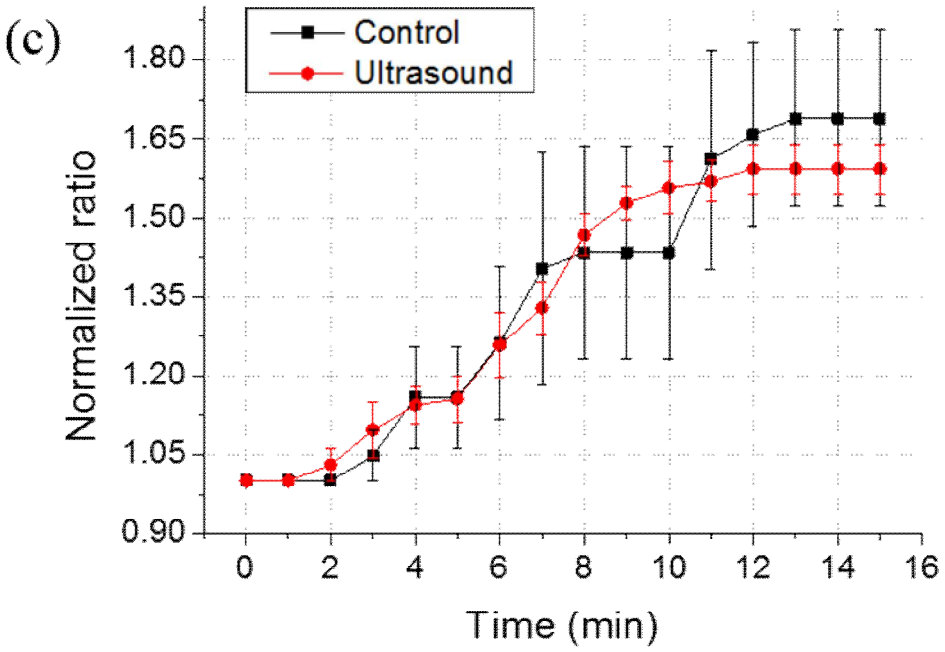


Figure 3.6-2. Normalized ratio of circular cells and control and ultrasound different height of medium, (a) 1.875mm, (b) 2.000mm.



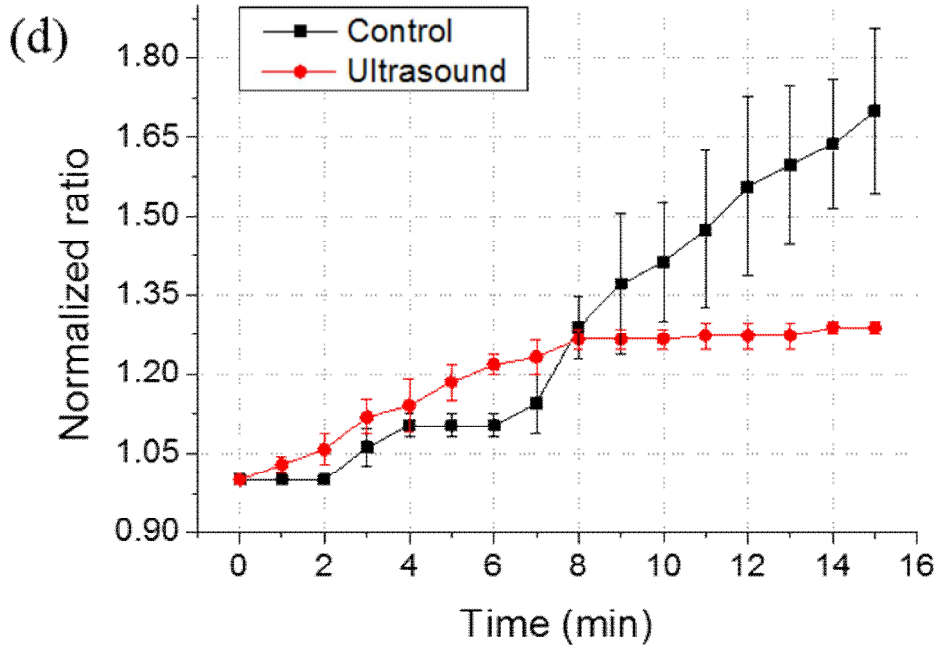


Figure 3.6-3. Normalized ratio of circular cells and control and ultrasound different height of medium.

(c) 2.125mm, (d) 2.250mm

IV. DISCUSSION

Ultrasound-induced cell stimulation is widely investigated during the last decade as a novel concept to manipulate at the cellular level. The mechanisms are very complicated and have yet not been explored in depth. One of the reasons is that there are too many experimental parameters affecting the results, which sometimes cannot be controlled (Yu and Xu 2014). Some parameters like the volume of culture medium may have large effects on the power of ultrasound irradiated to the cells (Hensel et al. 2011, Choi et al. 2013). In this study, we have attempted to demonstrate the simple one dimensional plain wave prediction for the standing wave field formed in a culture medium of the well, stating that acoustic pressure to the cells sitting on the bottom of the culture well will vary dramatically depending on the height of the medium.

HT-22 cell lines cultured in the incubator are subject to different survival environment when they are placed at the room temperature setup for the ultrasound stimulation experiment. Cells are most likely to respond negatively to their biological regulation systems and start to undergo changes in their morphology depending on the extent of the environmental and physical affects. We have observed that the cells exposed at room temperature environment start to diminish in their body along with the decrease of the neurites length in a relatively short time period, i.e. less than 15 minutes. We were able to foster the negative response of the morphology by means of impinging the ultrasound radiation as an extracellular physical stimulation. The temporal retraction ratio with respect to the initial length of the body demonstrated this effect with low intensity ultrasound (50mW/cm^2, continuous wave) even though this trend has to be confirmed with more results for higher ultrasound power.

In this study, we have attempted to investigate the standing wave effect on the cell morphology response depending on the medium height that produces different ultrasound power at the bottom of the well where the cells are located. It is shown that the HT-22 cell neurite more rapidly compressed to the cell body along with the cell body shrinkage, which resulted in the alteration of the cell outline into the

variation of the acoustic pressure against the culture medium height (Figure 2.3) is characteristically similar to the degree of the morphological cell responses (Figure 3.5 (b)). For the same nominal intensity ($50mW/cm^2$), the cell responses are different for the different culture medium height. As reported by Hu et al. (2013), certain acoustic output power resulted in instances of cell body displacement and the extent of the effects was dependent on acoustic intensity. The present observation may explain why the biological response of the cells to the ultrasound observed to be different for the same ultrasound power when the medium height was not controlled properly.

V. CONCLUSIONS

In this work, we have performed the first *in vitro* experimental demonstration at room temperature of the real-time dynamic response of the neuronal cell morphology to the ultrasound irradiation. We took advantage of the fact that the cells exposed to the room temperature will retract naturally due to the uncomfortable environmental conditions. The ultrasound energy delivered to the cells has promoted the morphological retraction ratio compared with the cells without ultrasound irradiation. In addition, the ultrasound intensity varies from 0 to the twice of the incident wave intensity at the bottom surface of the well where the cells are located depending on the medium height due to the standing wave effect. We have demonstrated that the variation of the acoustic peak pressure against the medium height (Figure 2.2) is characteristically similar to the degree of the morphological cell responses (Figure 3.5 (b)). This indicates that the ultrasonic exposure will be significantly different for the same ultrasound power generated from the transducer. The study claims that the parameter such as the culture medium height closely associated with standing wave effects must be monitored when *in vitro* ultrasonic cell experiments are performed. Further investigation which includes the physiological reactions of the cells is suggested to underpin the present results.

Acknowledgement

This master thesis has been carried out at the Department of Biomedical Engineering, Jeju National University, since autumn 2014. A number of people deserve thanks for their support and help. It is therefore my greatest pleasure to express my gratitude to them all in this acknowledgement.

I would like to convey my warmest gratitude to my supervisor Professor Min Joo Choi, who gave me the opportunity to conduct my study in his research group, support to me his **최민주교수 과제** (2013/9-2015/8) and for his guidance, generous contribution of knowledge and experience, valuable comments and encouragement from the start until the end of my study.

I also need to thank is supported by 외국인 장학금(학비), and 코러스트 인건비 지원(2016. 1~8).

My sincere thanks also goes to Professor Su-Yong Eun for offering me the 2 semester internship opportunities in their groups and I had been supported by 은수용 교수님 과제 (2016,9~2016,12).

My deepest gratitude also goes to Mister Gwansuk Kang and Seungmoo Yang who was very generous with their time, giving priceless knowledge and assisted me in each step to complete the thesis. Further I also would like to remind their support, kindness and patients.

I wish to express my deep thank to Dr. Hun Yun Hua for guiding me to study in South Korea.

I am especially indebted to all the members in my laboratory, Son Chang Kim, Oh Bin Kwon, Lee Ji Hyung and Jeon Sang Chan, their kindness, cooperation and assistance during my study.

Also, I greatly acknowledge to my friends Dash Oyunbileg, Amarsanaa Hulan and Ulziisaikhan Jawzandulam who study in Jeju National University for their valuable support which was much needed for the completion of this research.

Last but not least, I would like to thank my family for all their love and encouragement. For my parents and grandmother who raised me with a love of science and supported me in all my pursuits.

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