Recent Trends in Giga-Hertz *In-vivo* Acoustic Imaging for Biological Specimens: A Review.

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Abstract

There are a number of articles in literature available that discuss the combined effects of acoustic high-pressure and heat caused by acoustic vibration on biological tissues and cells. However, in this article, results obtained from separate application of acoustic pressure and heat, are provided. For finding the heat effect, a container including living human skin cells and a culturing medium on the X-Y stage equipped with the heating plate with temperature controller of the Scanning Acoustic Microscope (SAM) was located. Then, the temperature of the culturing medium was gradually increased, and *in-situ* observation was carried out. High power acoustic impulses by four methods, namely, High power laser pulse, Ultrasonic liquid and solid horns and an air gun (blunt impact). The cells were observed with the SAM just after giving the impact. The difference between phenomena indicating cellular insult and injury (*e.g.*, shrinkage or lift-off) were clearly visualized by the SAM with frequency at 1.0 GHz. Confocal laser microscopic images of the blunt impact specimens were also obtained.

1. Introduction

Human responses to blunt impact relate to a variety of fields ranging from sports injuries to biological cellular injuries. The approach was to employ living human skin cells as the smallest elements for test. The cells were grown, and maintained by giving proper “food” (*e.g.*, chemically defined cell culturing media containing a complex mixture of vitamins, sugars, serum proteins and the like). The cells were also grown on appropriate substrates under the controlled conditions of pH and gas (*i.e.*, mixture of O_2 (95%) and CO_2 (5%)). These preparations made it possible to compare the responses caused by the different factors in the following experiments.

First, thermal radiation was emitted to the cells to observe their thermal responses (*e.g.*, a physical deformation and a lift-off), wherein a mechanically scanned reflection acoustic microscope (SAM) was used to visualize the responses. Second, a blunt impact caused by ultrasonic pulses was given to the cells to observe the cellular mechanical responses, wherein the impact was intended to be a one-shot event during the time of a fraction of a second to avoid generating heat. Then, the SAM was used again to visualize the response of the cells. Images of the cells after the blunt impact by air gun pellet were also obtained using Confocal laser microscope.
The specimens were systematically injured so that the degree of injury could correlate with the severity of the insult. Furthermore, the distribution of injury rate of the cells was obtained by flow cytometry. The analyses of the responses may provide the fundamental data for estimating injury in human beings.

2. Experimental setup and procedure

2.1 Thermal insult

The human skin cells (*i.e.*, HaCat) were grown on the substrate (*i.e.*, sapphire) mounted on the bottom of the well with a culturing medium (*i.e.*, Dulebecco’s modification of Eagle’s medium: 1x (Mod)). The well was located on the X-Y stage having a function of the heating plate with temperature controller included in the SAM. The temperature of the culturing medium was monitored by the thermocouples.

2.2 Shock wave insult

There were four methods that were available for inflicting shock waves onto the cells, *i.e.*, a PZT plate radiating through a liquid horn, a PZT plate radiating through a solid horn, laser induced ultrasonic shock wave system and an air-gun system. The temperature was monitored by thermocouples to make sure that the heat generated was not significant.

An air gun shoots a substantially spherical (diameter – 5 mm) aluminum pellet, wherein the velocity of the bullet is controlled by the amount of air used for shooting. The aluminum bullet impacts the polymer/tungsten plate, located above the living cells grown on the bottom of the container (i.e., thin semi-transparent polymer membrane), which is located on the surface of a 200 kHz Panametrics transducer, which converts the shock wave into an electrical signal. The container is supported by a Plexiglas member to prevent movement from shock caused by the bullet impact. The amplitude of the electrical signal is measured and monitored by a Tektronix digital oscilloscope.

The center frequency of the ultrasonic wave is substantially 200 kHz. Using the calibration curve, the free-field voltage sensitivity is 203dB at 200 kHz. As the result of the hydrophone calibration, we obtain the following Equations. (1) and (2)

$$\begin{aligned} 203 &= 20\log_{10}\frac{V}{V_0} \\ \therefore \quad \frac{203}{20} &= \log_{10}\frac{V}{V_0} \qquad (1) \\ \therefore \quad 10.15 &= \log_{10}\frac{V}{V_0} \end{aligned}$$

$$1V_0 = 1\mu Pa \qquad (2)$$

Pressure (P) is expressed in the following Equation. (3)

$$P = V \times 10^{\frac{203}{20}}\mu Pa = \left(10^{4.15}\right)VPa \cong \left(1.413\times10^{4}\right)VPa \qquad (3)$$

Pressure is also expressed as force in the following Equation (4)

$$1Pa = 1N/m^2 \qquad (4)$$

Area of the tungsten plate is 25π mm^2. Therefore,

$$F = \left(1.413\times10^{4}\times25\pi\times10^{-6}\right)N/V \cong 1.1N/V \qquad (5)$$

The Air-gun pellet impacted specimens were then stained to be observed under a Confocal Laser Microscope. After impact and removal of the media, the specimens were rinsed with Phosphate Buffered Saline (PBS). 4% Para formaldehyde (in PBS) was added to fix the cells at room temperature. Cells were then washed 3 times for 5 minutes with PBS. Then, 0.1% Triton X-100 (in PBS) was added and 1% Bovine Serum Albumin (BSA) (in PBS) was added. To this, phalloidin at a 1:50 dilution and SYBR Green at a 1:5000 dilution in 1% BSA was mixed. The whole mixture was then incubated with Antibody/BSA solution for 20 minutes. Finally, the fixed cells were washed 3 times for 5 minutes with PBS.

3. Results

3.1Thermal insult

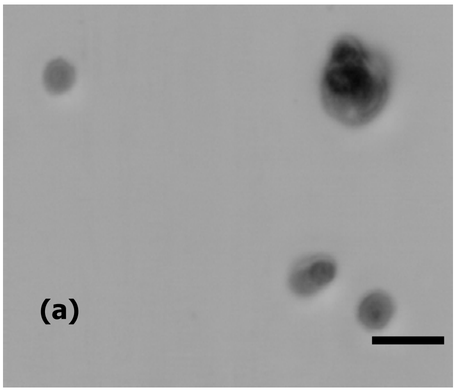
The changes in the behavior of the living cells due to the elevation of temperature (37.5~52.0°C) in the culturing medium was observed. When heating the living healthy cells, the cells would gradually die through the processes of the physical deformation (*i.e.*, shrinkage of the cells and decrease of the contact area of the cells and the substrate), lift-off and float. The lift-off cells were found to be badly injured, and statistically hardly recovered. Therefore, to find the lift-off cells was critical to discriminate between the dead and living cells.

The SAM images show clear effects of increasing temperature, from 37.5 to 44.5°C. The images of shrinkage, delamination, and death of human skin cells are obtained. (a) The image of healthy cells at 37.5°C; (b) The image of the shrunk and lifted off cells at 42.5°C; (c) The image of the cells, wherein some dead cells moved from the group at 44.5°C. The images were formed with frequency at 1.0 GHz. The acoustic lens was defocused at $z = -1.6\mu m$.

Another set of human skin cells were grown on the slide glass located on the bottom of the Petri-dish with 1x(Mod) of Dulebecco's modification of Eagle's medium to find shrinkage rate in accordance with the elevation of temperature of the medium. The temperature was increased from 38.5 to 52.5°C. It was found out that more the temperature, more the shrinkage is.

3.2 Acoustical impact

Figures 1(a) and 1(b) show the healthy cells and the cells impacted by shock waves, respectively. As can be appreciated, all impacted cells in the region were lifted off. Fifty pulses in a ten seconds interval at full power were necessary to destroy the cells. The lift-off cells were stained with TRYPAN-BLUE (0.4%) solution. A laser scanning microscope was then used to confirm that the lift-off cells were dead.



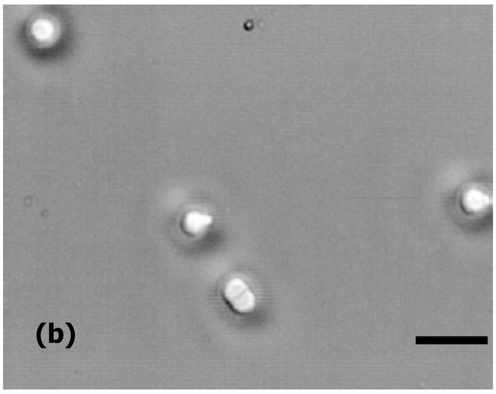


Figure1. SAM images show that the cells subjected to mechanical insults remained flat on the substrate. The acoustic lens was defocused at z = -1.6µm for obtaining both images. The bar is 50 µm. (a) Healthy cells at 37.5°C; (b) Impacted Cells at 37.5°C.

4. Conclusions

Scanning acoustic microscopy was found to be a useful tool for visualizing injury to skin cells. Clear differences could be observed between thermal and shock wave insults. In-vivo images observed with frequency at 0.6 GHz and 1.0 GHz by SAM show clear effects (*i.e.*, from shrinkage to lift-off) of increasing temperature. SAM images show that cells subjected to mechanical insult had little physical deformation or shrinkage and were damaged to the point of complete lift off. Bullets from an air-gun were shot onto the cells to provide blunt impact. Confocal laser micrographs showed that the cell layer thickness increased after the impact but decreased in time. The staining of the blunt-impacted cells took 30 minutes. Therefore, immediate response of the cells was not known. Further work needs to be done to accurately determine the cell recovery processes with respect to time. More detailed studies at lower doses of shock wave intensities and pulse numbers are required to determine the full scope and range of response to shock wave insults for human skin cells.

It was observed that the cell layer thickness initially increased in height (60 minutes), after the blunt impact and decreased later in time (90 minutes). For a typical specimen, the measured height after fixing (30 minutes) was 7 µm which then increased to 9.875µm after 60 minutes and then decreased to 4.875 µm, after 90 minutes.

5. References

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