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Frequency dependent Shape transitions in Micro-confined Biological cells

Hritwick Banerjee
Research Engineer/Department of Biomedical Engineering
National University of Singapore, Singapore

***Abstract*— To demonstrate the therapeutic potential of low-intensity ultrasound, it is imperative to characterize the bio-physical interaction of living cells with alteration of ultrasound frequency which was an obscure topic thus far. Here, we used a microfluidic platform for *in situ* single cell analysis with bio-physical interaction to ultrasound frequency alteration in line with the fact that microfluidic channels to a large extent mimic the confinement effect induced by micro confinement of physiological pathways. In this article, with the help of series of single-cell direct observation, we show that low intensity ultrasound frequency alteration would reversibly perturb cell membrane structure and count for inherent cell oscillation. However, during post exposure ultrasound period the cytomechanical perturbation of cell membrane is relatively more compared to ultrasound exposure period leading to an inherent residual strain which follows a transition zone near to the resonating frequency of the composite system. Together, these findings indicate that alteration of low intensity pulsed ultrasound (LIPUS) frequency, if applied to a microfluidic platform on the order of minutes, would produce a reversible effect on physical structures of living cells based on the system resonant frequency during and post exposure ultrasound pulsing.**

Index terms -Cellular Morphology, Cytomechanical, Microfluidic Confinement, LIPUS.

I. INTRODUCTION

LIPUS is a well-established platform for clinical treatment of fractures and other osseous defects, which already been demonstrated to accelerate *in vivo* all stages of fracture repair process (inflammation, soft callus formation, hard callus formation) [1]. Level I clinical studies demonstrate the ability of a specific ultrasound signal (1.5 MHz ultrasound pulsed at 1 kHz, 20% duty cycle, 30 mW/cm^2 intensity, Spatial Average and Temporal Average Intensity (SATA)) to accelerate the healing time in fresh tibia, radius and scaphoid fractures by up to 40% [1]. LIPUS induced mechanical deformations activate receptors on cell membranes such as integrins, mechanosensitive-calcium channels, G-proteins, IGF, TGF-β/BMP and gap junctions, activating different downstream pathways [1]. Nevertheless, current evidence on the treatment efficacy of LIPUS is often considered to be controversial in the evidence of human trials in fresh, stress fractures and limb lengthening as the extent of therapeutic effects varies greatly [2-3].

Although useful therapeutic effects are now being demonstrated clinically, the mechanism by which the sequence of cytomechanical events involved because of wave-matter interaction is not clearly understood. However, only a fragmented finding is being reported in this topic. A general trend that has been observed is post-exposure cytoskeletal remodelling [4-7], and it may result in the alteration of the cell's adherence characteristics [8-10]. While ultrasound-induced alterations in downstream cellular functions have been confirmed by these studies, it remains unknown as to how ultrasound would physically perturb cells. In this context Hu et al. 2014 [11] presented a quantitative analysis by acquiring direct observations of cytomechanical dynamics which reversibly perturb the physical and subcellular structure of living cells during and after low-intensity ultrasound exposure. However, the detailed depiction of the effect of altering ultrasound frequency on cellular morphology was trivial from their initial study. Without an in-depth investigation into this upstream course of action, it would be difficult to formulate a coherent biophysical description of the cytomechanical events induced by alteration of ultrasound frequency of application.

Here we bring out nontrivial influence of pulsed frequency on the cellular morphologies subjected to perturbation imparted by low intensity ultrasound exposure. Instead of open system we used a confined microfluidic platform, in line with the consideration that microfluidic channels to a large extent mimic the confinement effects induced by micro confinements of physiological pathways. Our investigation is founded on the hypothesis that the mechanical bio-effects of LIPUS frequency alteration are fundamentally initiated through physical perturbations of the cellular structure. To further reinforce the hypothesis, we unveil how LIPUS alter cell membrane morphology at different frequency of application. To counteract the study further we used osteoblast cell line, MG63 for mechanoresponsive study. It should be emphasized that the notion of alteration of low intensity ultrasound frequency has not been clearly defined in medical ultrasound community; we restrict our frequency range 1-3 MHz which happen to exert its biological effects via a non-thermal, as yet undefined biochemical action [12-13]. We executed all of our experiments within the regulatory limits set by the U.S. Food and Drug Administration as normative thresholds: (i) 0.72 W/cm^2 for spatial-peak time averaged (SPTA) intensity, and (ii) 190W/cm^2 for spatial-peak, pulsed averaged intensity [14].

By series of single cell direct observation, we show that alteration of LIPUS frequency of application between 1-3 MHz, if applied to a glass-PDMS microfluidic platform in the order of minutes would reversibly perturb the cell membrane structures and inherently called for cell oscillation based on the system resonant frequency condition.

II. MATERIALS & METHODS

Experimental apparatus for ultrasound-cell interaction analysis.

A. Hardware description

Our investigation for ultrasound-cell interaction analysis was conducted with three main integrated hardware units (i) an ultrasound transmission setup whose waveform parameters can be arbitrarily defined; (ii) a hybrid glass-PDMS microfluidic device which contained a linear microchannel with rectangular cross section; (iii) confocal microscope that supported live in-situ imaging of cellular response over the course of ultrasound exposure. Figure 1 describes the block diagram of these three hardware units, components and how they were connected in a pictorial form.

As illustrated the ultrasound transmission setup consisted of serially connected function generator (33220A 20 MHz Function/Arbitrary Waveform Generator, Agilent Technologies) and ultrasonic transducer (Pz26, FERROPERM PIEZOCERAMICS A/S). During operation, the ultrasound-transducer emitted pulses based on waveform parameters defined by the function generator. These pulses then passed through the glass-PDMS interface and entered into linear microchannel which was seeded with cells. The cellular response to frequency alteration of ultrasound pulsing was then monitored in situ using the confocal microscope (FV 1000 Fluoview, Olympus).

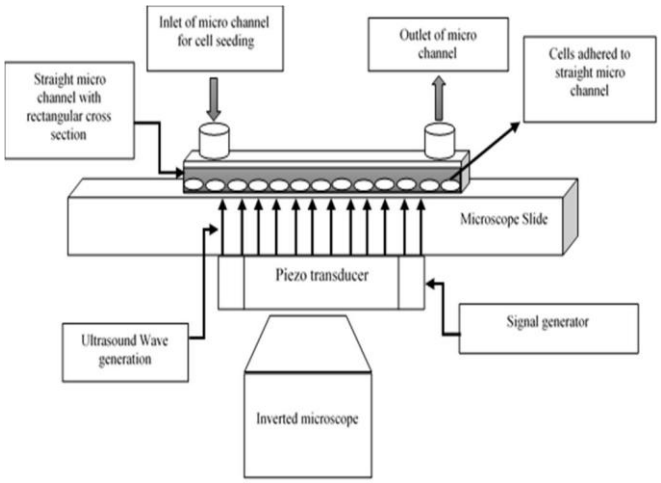

Figure 1. Schematic of the experimental setup showing ultrasound generation and transmission to the micro channel through microscope slide into adhering cellular surface. A standing ultrasound wave naturally arises due to reflection off the edges of the substrate. If a propagating surface acoustic wave (SAW) is desired, α-gel is placed along the edges to absorb the radiation, preventing reflection.

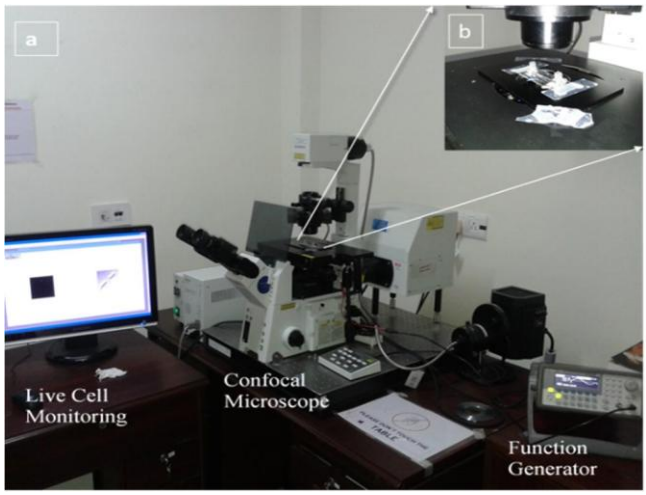

Figure 2. (a) Complete experimental setup for ultrasound-cell interaction analysis through single live cell imaging on the stage of confocal microscopy. (b) The inset of glass-PDMS hybrid microfluidic device containing a linear channel with rectangular cross-section mounted on the stage of confocal microscope.

B. Micro channel Preparation

With view of mimicking the confinement effect that resembles the downstream physiological pathway we used a microfluidic platform for single cell analysis influenced by ultrasound exposure. Along with many other potentials this microfluidic systems created precisely defined microenvironments by controlling fluidic and surface chemistries, feature sizes, geometries and signal input timing, and thus enabled quantitative multi-parameter analysis of single cells.

The PDMS microfluidic device (channel width: 1.5mm; height: 100μm; length: 4cm) was fabricated with Polydimethylsiloxane (PDMS) polymer using a standard Soft Lithography technique [15]. The PDMS layer containing a linear microchannel with rectangular cross section was then plasma bonded with piranha-cleaned glass slide.

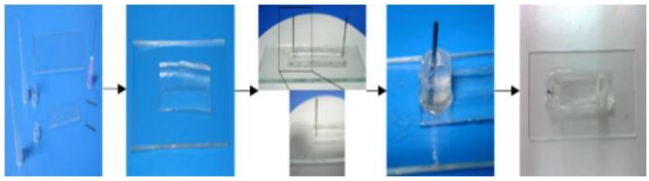

Figure 3. Step by step procedure for fabricating glass-PDMS hybrid microfluidic device which contains a linear channel with rectangular cross-section.

C. Cell type and culturing

In the present study, human osteoblast cell line, MG63, was used as a model mechanoresponsive cell and

cultured using following standardized protocols [16]. The MG63 cells were first cultured to 70% confluence within a 75-cm^2 culture flask that was placed inside an incubator with 37^0C temperature and 5% carbon dioxide (CO_2). Minimal Essential Medium (D-MEM, HyClone) supplemented with 10% heat-inactivated fetal calf serum (FBS, HyClone) was used as the culture medium. The cultured MG63 cells were harvested by adding 1 mL of 0.25% trypsin into the culture flask after removing the culture medium and washing three times with phosphate buffered saline (PBS). They were then transferred to the micro channel at a seeding density of 10^6cells/mL. To foster attachment of these cells to the substrate, hybrid glass-PDMS device was placed inside the incubator for 24 hr in the presence of culture medium.

D. The piezoelectric setup

Ultrasonic transducer Pz26 (FERROPERM PIEZOCERAMICS A/S) was chosen as it is an all-round hard PZT material with good coupling factors, high curie temperature, high mechanical quality factor, low dielectric loss and a very good stability over time. There after Pz26 was cut (1 cm × 1.2 cm) and designed to fit the breadth of the microscope slide. Then silver gel was coated to differentiate positive and ground terminal to prevent short circuiting and placed in the hot air oven at 50^0C for 30 minutes. There after the thin cupper wires were cut and soldered with Pz26 to enhance externally triggered electrical input. To check the working state, a function generator with a sine wave of amplitude 10 V peak to peak and 10 kHz frequency were applied to the newly made PZT.

The newly made PZT was then glued with 98% glycerol and coupled with back side of glass-PDMS *hybrid* microfluidic device for ultrasound exposure.

Figure 4. (a) The piezoelectric transducer of dimension: 1 cm × 1.2 cm. (b) Transducer placed and coupled in the back side of microfluidic device.

E. System resonant frequency calculation

To determine the resonant frequency of the hybrid glass-PDMS device we at first connected a T-connector to the oscilloscope (DSO1012A Oscilloscope, 100 MHz, and 2 Channel Agilent Technologies) input. The two free ends of T-connector were then connected with (i) two wires of PZT which was further connected through the glass-PDMS device (ii) the output of signal generator. Before switching on the signal generator the glass-PDMS device was filled with deionized distilled water (Milli-Q). Pulse frequency of 1MHz-3MHz with 30mW/cm^2 spatial peak time averaged ultrasound intensity and burst rate of 1 kHz at an interval of 200μs were delivered through the function generator and the output waveform had been constantly monitored on the oscilloscope. During frequency sweep it was observed that near to 1.7 MHz there was a minima of peak to peak voltage wave while if we move to lower/higher frequencies the voltage sharply increases. To conclude the fact that in between 1MHz-3MHz pulse frequency range the composite glass-PDMS system exhibited its resonance near to 1.7 MHz.

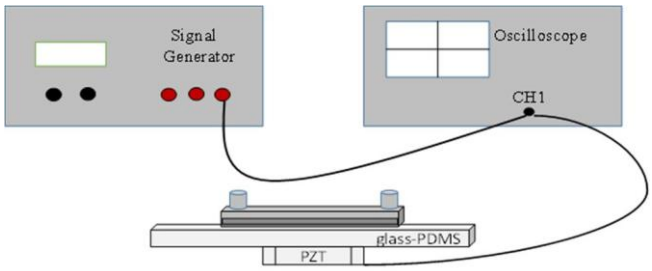

Figure 5. The schematic of the setup to measure system resonant frequency. An oscilloscope is connected to signal generator and hybrid glass-PDMS device through a T-connector.

III. RESULTS & DISCUSSION

A. Low intensity ultrasound produces two sided cell size reduction

Progressive change in cell size was observed during the exposure of low intensity pulse ultrasound (LIPUS). Figure 6. illustrates the live cell imaging in confocal microscope of MG63 morphological dynamics during and after exposure to 1MHz pulse frequency with 30mW/cm2 ultrasound intensity and burst rate of 1 kHz at an interval of
200μs. It may be noted that during 3min 45sec ultrasound exposure time, MG63 cell exhibited membrane expansion in time lapse basis. However, the cell membrane expansion was transient in nature as during the post exposure time the cell membrane shrank and tried to recover its original cellular morphology. It can be observed that 3min 45sec after switching off the LIPUS the sonicated cell tried to come back to their pre-exposure size and stopped in a lower membrane area scale producing a plastic deformation.

For the sonicated cell depicted in Figure 6. some trivial changes in cross-sectional area were found during exposure to LIPUS while there was a significant cross-sectional area change observed after switching off the LIPUS in Figure 7(a). Here ultrasound pulsing the cell cross-sectional area increase was trivial and the time lapse size recovery during post exposure of LIPUS was significant. However, during post-exposure time the cross-sectional area shrank even below the original pre exposure cell cross sectional area. The uniformity of this phenomenon is illustrated in Figure 7(b) in which normalized Arial area strain during and after ultrasound exposure is plotted in a temporal scale.

It is worth noting that the rate of size increase during ultrasound exposure was lower than the rate of size recovery during post exposure time. To quantify such a trend, we used a quadratic fit of the cell cross-sectional area data in a scatter plot during and post ultrasound time separately. For this we took the time instant between T 0:00 to T 02:48 for both during and after LIPUS exposure.

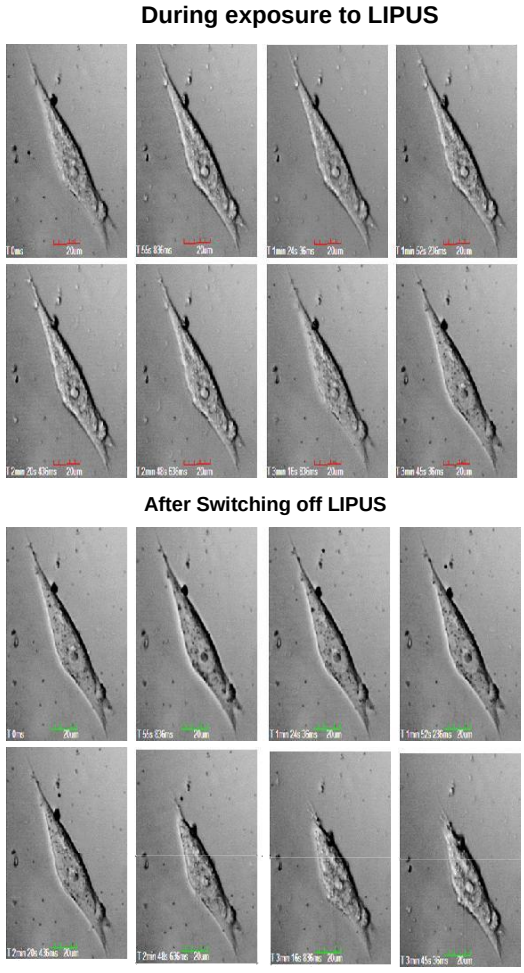

Figure 6. Time series bright field images showing cytomechanical changes of MG 63 cells during 1-MHz ultrasound pulsing with 30mW/cm2 spatial peak time averaged ultrasound intensity and burst rate of 1 kHz at an interval of 200µs for 3min 45 sec time duration. Time-lapse cell shrinkage is evident during post exposure ultrasound period while cell expansion during ultrasound exposure is relatively less prominent.

B. Cell morphology at system resonating frequency

As previously discussed the system resonating frequency is nearly at 1.7 MHz, during which it presumes to exert maximum amount of mechanical vibration into the system as seen in Figure 8. Here in figure 8. we observed a trivial change during ultrasound exposure while during post exposure ultrasound period the cell shrinks progressively and tries to detach from the surface of micro channel. There are two probable reasons behind the detachment effect caused in the cellular morphology. One, due to the action of strong acoustic radiation forces directed away from the wall and secondly due to apoptosis.

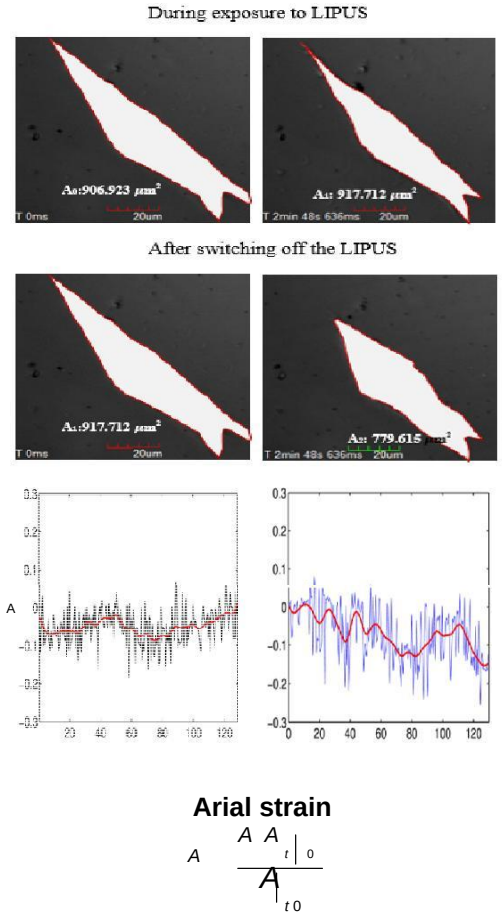

Figure 7. (a) Schematic illustration of changes in cellular cross-sectional area from T 0:00 to T 2:48 sec during and post exposure ultrasound pulsing. (b) Arial strain during and after 1-MHz ultrasound exposure period.

This fitting technique resulted in a cell cross-sectional area expansion by 1.18% during ultrasound exposure while a sharp decrease by 15.05% observed after switching off the LIPUS. This difference of percentage change of cross sectional area was even traced in the cellular morphological change in the Figure 8(a).

This further introduces the negative effect of adherence property during post exposure ultrasound pulsing. The cellular detachment due to apoptosis and progressive shrinking effect during resonant frequency condition is adverse and produces detrimental effect of LIPUS. So for further investigation we restrict our experiments into two regimes- 1. Below resonating condition 2. Above resonating condition.

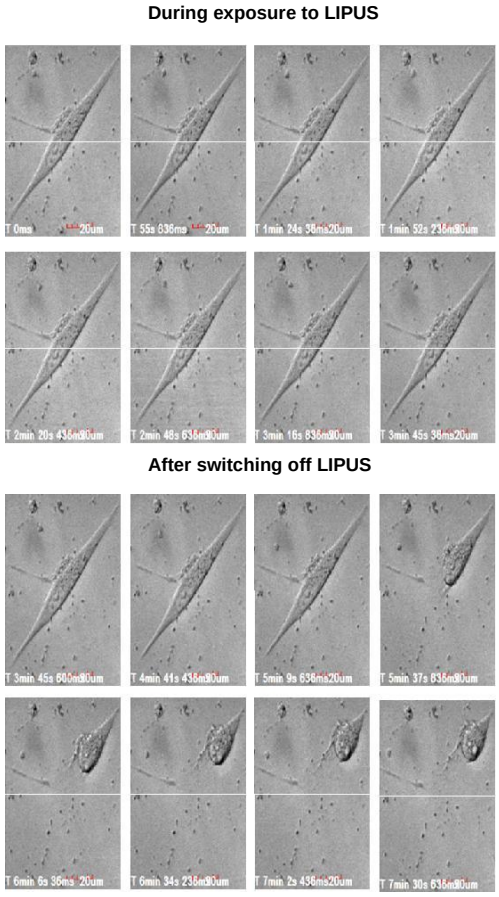

Figure 8. Time series bright field images showing cytomechanical changes of MG63 cells during 1.7-MHz ultrasound pulsing with 30mW/cm^2 spatial peak time averaged ultrasound intensity and burst rate of 1 kHz at an interval of 200µs for 3min 45 sec time duration. Time-lapse cell shrinkage is evident during post exposure ultrasound period while cell expansion during ultrasound exposure was relatively less prominent.

C. Cell morphology change during alteration of ultrasound pulsing

We examined the cell morphology change while the ultrasound pulse frequency was altered from initial 1MHz to 2.5MHz in an increasing step of 0.5 MHz whereas the burst rate of 1 KHz at an interval of 200µs remained the same. There was an interesting cell morphological change observed during alteration of frequency as presented in Arial strain plot (Figure 9.). With accordance to the 1MHz ultrasound frequency we performed similar fitting technique for other higher ultrasound frequencies during and after ultrasound exposure. The data points of relative area change in percentage obtained from post processing image analysis in MATLAB software. Figure 10. (a, b) depicts a simple bar chart of cell surface area change for during and post ultrasound exposure period of different pulse frequency of application.

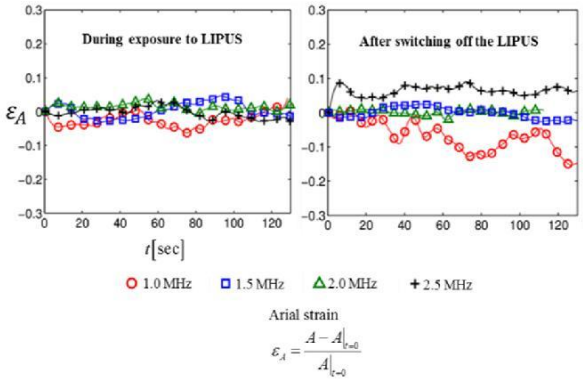

Figure 9. Arial strains during and after switching off LIPUS for different ultrasound frequency of application.

On the other hand, the reverse phenomenon observed during post exposure ultrasound period as the sonicated cell tries to revert back to its original shape as can be shown in Figure 10. (b) clearly. But one key point to be mentioned is that even if during post exposure time the cell tries to catch its original shape there is always a mismatch of cross-sectional area between the first time the ultrasound is been exposed and the end of post exposure period.

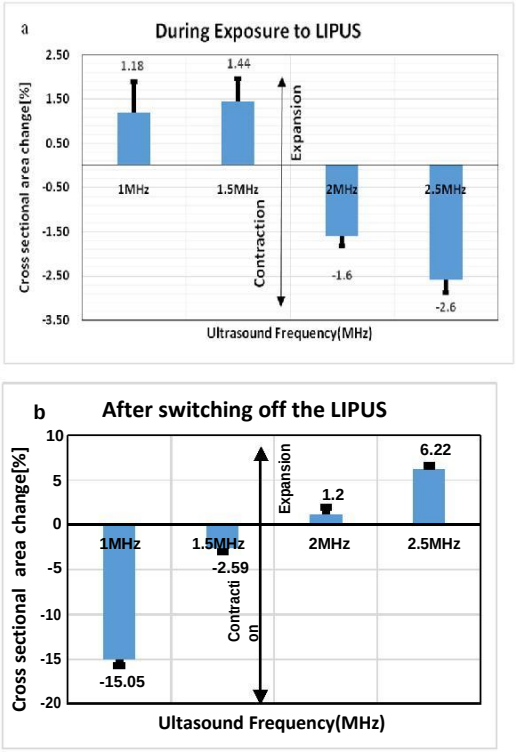

Figure 10 (a, b). Bar chart with error bars of cell cross sectional area change (%) from its previous morphology during and after switching off LIPUS. The value of relative percentage area change is tied with the frequency bar. Here

positive value represents area expansion while negative (-) value signifies area shrinkage/contraction

Cellular morphology mismatch between pre and post ultrasound exposure period generally refers to a plastic deformation of the cellular morphology. This hypothesis is even traced while we have exposed the hybrid glass-PDMS device again to ultrasound second time after the post exposure period. During second time of ultrasound exposure hardly any change in cellular morphology been traced as the plastic deformation is considered to be permanent in nature (Figure 11.).

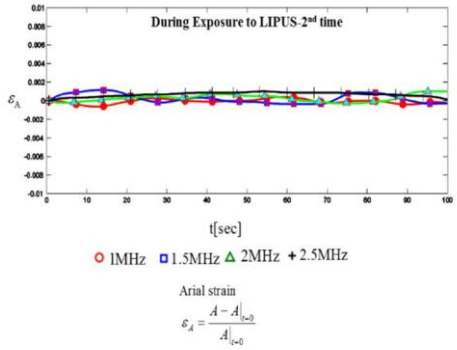

Figure 11. Arial strain during exposure to LIPUS for second time after post exposure period to unveil the phenomenon of plastic deformation.

D. Amplitude of Arial strain during ultrasound alteration

The experimental results of cell morphology change during alteration of ultrasound frequency further instigates the established phenomenon of cellular membrane intrinsically capable of absorbing mechanical energy from ultrasound field and yield expansion and contraction in the transmembrane place[17]. Scaling analysis of our result using an inverse square root fitting described in below figure is mimicking the hypothesis of Krasovitski et al. 2011[17], which states that the amplitude of maximum area strain is inversely proportional to the square root of ultrasound frequency of exposure if acoustic pressure remain nearly constant ($A \sim P_A^{0.8} f^{0.5}$).

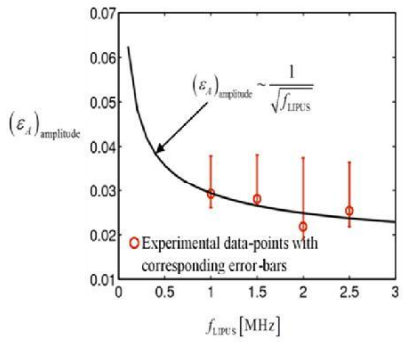

Figure12. Amplitude of areal strain during the exposure of the cell to the LIPUS

Here we have also tried to unveil the fundamental question of whether the cellular expansion and repression oscillation during and post exposure ultrasound period is inherent in nature or contrast agent (UCA) is necessary. The sonicated cells oscillation during and post exposure ultrasound period are mainly governed by elasticity of the cell membrane and the viscosities around the medium [18]. This membrane oscillation and cell area deformation during a dipole and quadrapole also depend on the surface area modulus. This phenomenon of cell surface area change used to get enhanced by inclusion of contrast agent microbubble and develop its maximum peak near the resonating frequency. However, the peak of surface area deformation near the resonant frequency is not high enough to produce a significant mechanical effect and might rupture the cell in the vicinity of oscillating microbubble [18].

E. Residual Arial strain during post exposure period

Our experimental findings necessarily support the established phenomenon of expansion and repression of cell cross sectional area during and post exposure ultrasound period. During restoration of pre-exposure cell morphology, we found that there is a residual strain still exists after 2min 48 sec of time live imaging. This occurrence supports the fact that cell tries to recover its transient change of membrane cross sectional area deformation in lieu to the mechanical stress generated by ultrasound wave. However, it should be noted that the existing residual strain might lead to a plastic deformation even after the recovery time lapses. During frequency alteration as we move to higher frequency regime [>2 MHz] there is a transition zone observed in Figure 13 which is closely in the domain of resonating frequency of the system (nearly 1.7 MHz). As the frequency of ultrasound increases the wavelength decreases which lead to lower penetration depth [19]. While the penetration depth is lower for higher frequency the possible reason of transition from shrinkage to expansion during post ultrasound exposure may occur as in our case.

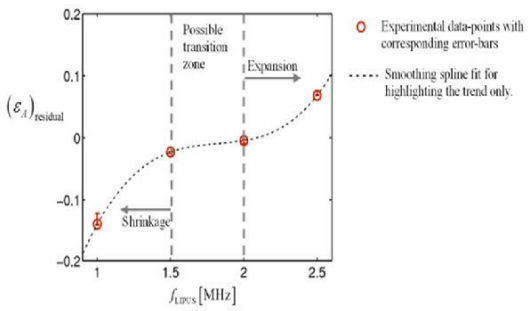

Figure 13. Areal strain that remains after 2 min 48 sec (approx .) from switching off the LIPUS.

Our findings are significant to cellular development as it is a recognised fact that the perturbations of cell's

mechanical microenvironment at the membrane interface [20] would alter cytoplasmic signalling and gene transcription processes that in turn, would modulate various cellular behaviours including lineage determination, migration and motility [12]. Thus our investigation serves to uncover the cellular process to induce potential therapeutic effects during frequency alteration of low intensity ultrasound domain.

F. Relevance to low-intensity therapeutic ultrasound research

In this article we investigated the effect of alteration of ultrasound frequency and change in cellular morphology which remained an obscure topic in low intensity therapeutic ultrasound research. The most important point to note is that the time duration of upstream morphological events observed in our experiments is less compared to the downstream simulatory bio-effects that lasts hours or days after exposure. So, a definite connection has to be made in this regard.

The further investigation of the above realm would readily supplement the existing findings of effective dosage levels in low intensity therapeutic ultrasound [12-13, 21-22]. For an example, in bone healing application, the fundamentally used 20 min a day daily of 30mW/cm^2 spatial-averaged time-averaged intensity (SATA) treatment regime calls for further investigation of cellular dynamics study and therein [4,23]. The key point is that mostly therapeutic ultrasound dosage were rudimentarily being investigated and thus far from optimal in acoustic intensities in situ [24-25].

IV. CONCLUSION

To unveil the fundamental question of how ultrasound enhance stimulatory bio effects come about, we carried our experiments in microfluidic platform by acquiring direct observations of cytomechanical dynamics of sonicated MG63 cells during and after ultrasound exposure for different pulse frequency of application. We experimentally demonstrate that alteration of low intensity ultrasound frequency, if applied to a glass-PDMS hybrid microfluidic platform on the order of minutes, would produce a reversible effect on physical structures of living cells based on the system resonant frequency during and post exposure ultrasound pulsing. Our study should pave way for further efforts to integrate the physical phenomenon of ultrasound pulsing and the current knowledge of mechanobiology. This further effort would undoubtedly constitute the foundation of mechanistic effects of low intensity therapeutic ultrasound and its allied potential in medical applications therein Our study should pave way for further effort to integrate the physical phenomenon of ultrasound pulsing and the current knowledge of mechanobiology. This further effort would undoubtedly constitute the foundation of mechanistic effects of low intensity therapeutic ultrasound and its allied potential in medical applications therein.

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Authors Profile

Hritwick Banerjee holds Master of Technology **(M.Tech)** in Electrical Engineering from Indian Institute of Technology (IIT) 2014. Currently, he is working as a Research Assistant in "Laboratory of Medical Mechatronics", Department of Biomedical Engineering; National University of Singapore (NUS). In the recent past, he worked as a Research Engineer at NUS in "Cardiovascular Biomechanics and Ultrasound Laboratory". Before this he worked as a Research Assistant at Indian Institute of Science (IISC) Bangalore in Center for Nanoscience and Engineering (CeNSE) department. His broad area of research interest is Bio-Microfluidics, therapeutic ultrasound, drug delivery and surgical tool development to touch upon the human lives from an engineering approach and beyond.