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Short Communication

Fluorescence response of human HER2+ cancer- and MCF-12F normal cells to 200 MHz ultrasound microbeam stimulation: A preliminary study of membrane permeability variation

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ABSTRACT

Targeted mechanical cell stimulation has been extensively studied for a better understanding of its effect on cellular mechanotransduction signaling pathways and structures by utilizing a variety of mechanical sources. In this work, an ultrasound-driven single cell stimulation method is thus proposed, and a preliminary study is carried out by comparing the fluorescence intensities representing a change in cell membrane permeability between MDA-MB-435 human HER2+ cancer cells (~40–50 μm in diameter) and MCF-12F normal cells (~50–60 μm) in the presence of ultrasound. A 200 MHz single element zinc oxide (ZnO) transducer is employed to generate ultrasound microbeam (UM) whose beamwidth and depth of focus are 9.5 and 60 μm, comparable to typical cell size. The cells in tetramethyl rhodamine methyl ester (TMRM) are interrogated with 200 MHz sinusoidal bursts. The number of cycles per burst is 5 and the pulse repetition frequency (*PRF*) is 1 kHz. The temporal variation of fluorescence intensity in each cell is measured as a function of input voltage to the transducer (16, 32, and 47 V), and its corresponding fluorescence images are obtained via a confocal microscope. A systematic method for visualizing UM's focus by adding Rhodamine B to the immersion medium is also proposed to enhance the precision in aiming the beam at an individual cell.

Both types of cells exhibit a decrease in the intensity upon UM irradiation. In particular, normal cells show more fluorescence reduction (down to 0.7 in normalized intensity) than cancer cells (~0.9) under the same excitation condition of the transducer. With UM being turned off, the normalized intensity level in normal cells is slowly increased to 1.1. The cell images taken before and after UM exposure indicate that the intensity reduction is more pronounced in those cells after exposure. Hence the results show the potential of UM as a non-invasive in vitro stimulation tool for facilitating targeted drug delivery and gene transfection as well as for studying cellular mechanotransduction.

1. Introduction

Dynamic cellular responses to externally applied forces that induce various aspects of cell behaviors [1] have long been of scientific interest to many researchers. Such an emerging interest has led to the development of a number of approaches in applying external forces to cells and tissue cultures for mechanotransduction studies [2]. In particular, electric stimulation of ganglion cells using multi-electrodes has offered a better understanding of how to activate neurons with extracellular electric stimuli applied to the retinal surface. Stimulation current varying from 0.6 to 16.7 μA has been applied to the retinal tissue (1–2 mm in diameter) separated from the pigment epithelium [3]. The effect of in vitro electrical stimulation on generation of reactive oxygen species and cardiogenesis in embryoid bodies derived from human embryonic stem cells has been investigated in a custom-built electrical bioreactor under the electrical field of 1 V/mm [4]. Transcranial magnetic stimulation has also been carried out to study its influence on electroencephalography (EEG) oscillatory activity in motor cortex of healthy human brain [5]. In order to reveal the relation between magnetic field and cortical excitability modulation,

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a maximum field of 2.2 T produced from a commercial magnetic stimulator (Magstim Company, Whitland, UK) has been applied to the primary cortex at 1 Hz for 10 min.

In contrast to complex experimental systems and high implementation cost of the aforementioned approaches, ultrasound can be an alternative stimulation tool, because it is simple and inexpensive to construct such systems. Low intensity (up to 400 mW/cm^2) ultrasound stimulation has been demonstrated to induce chondrogenic differentiation of mesenchymal stem cells in vitro for cartilage tissue engineering [6], whereas techniques for ultrasound-enhanced drug delivery has been developed with high intensity ultrasound (~2660 W/cm^2) [7]. Temporary pore formation in the membrane caused by ultrasonic irradiation has also been utilized to improve nanoparticle penetration into breast cancer spheroids [8] and chemotherapeutic efficacy in retinoblastoma cells in vitro [9]. In these low frequency (1–2 MHz) techniques, however, multiple cells have been simultaneously exposed to sound beams with the beamwidth of several hundred micrometers.

In order to promote individual cellular interaction with ultrasound, a narrow beamwidth approaching a certain cell size is crucial. This can be made possible as the frequency is increased to beyond 100 MHz at which the beamwidth may become as narrow as a few μm. Ultrasound of 100 MHz to a few GHz range is here termed as ultrasound microbeam (UM). As an application of UM, two-dimensional cell manipulation has been experimentally demonstrated with 200 MHz sinusoidal bursts (*PRF* = 1 kHz, duty factor = 0.025%, and supplied voltage to the transducer = 2 V), showing that the beam is capable of trapping a leukemia cell at the focus without affecting other neighboring cells [10]. Elastic properties of zebrafish embryos are parametrically mapped through acoustic radiation force impulse imaging at 100 MHz [11].

More recently, it has been found that tetramethyl rhodamine methyl ester (TMRM) fluorescence accumulated in mitochondria can be used as an indicator of mitochondrial membrane permeability [12]. Therefore it can readily be envisioned that the change in the cell membrane permeability resulted from targeted UM irradiation may be monitored by measuring the fluorescence intensity arising from an individual cell.

In this paper, a preliminary study to demonstrate the feasibility for the use of UM as a targeted mechanical cell stimulation tool is undertaken by measuring the fluorescence modulation of cancer (MDA-MB-435) and normal cells (MCF-12F) in the presence of TMRM. Those cells are non-invasively stimulated by a focused ZnO transducer at 200 MHz. The effect of UM interrogation on each cell is evaluated with respect to the fluorescence intensity by a confocal microscope. The temporal intensity changes of TMRM are compared between the cell groups, as a function of ultrasonic beam intensity represented by the supplied voltage to the transducer here, given that there exists no device that is capable of measuring ultrasonic intensity at such high frequencies (>100 MHz). A systematic approach for defining UM's focus is also devised by adding heat-sensitive Rhodamine B to the immersion medium, allowing more accurate exposure of a single cell to UM. The results are reported to show the UM's capability as an alternative means for targeted drug delivery and cellular mechanotransduction study.

2. Materials and methods

2.1. Cell preparation

MDA-MB-435 human HER2+ cancer cell (~40–50 μm in diameter) and MCF-12F normal cell (~50–60 μm) lines are obtained from the National Cancer Institute and ATCC (American Type Culture Collection), and maintained in DMEM, 10% fetal bovine serum under 5% CO_2. TMRM is purchased from Invitrogen (Grand Island, NY) and laser grade Rhodamine B is obtained from Acros Organics (Pittsburgh, PA). Carbonate buffer, 20 mM/L at pH 9.4, is also prepared to make Rhodamine B solutions (0.1 mM/L). Temperature around the cells is maintained at 37 °C in a delta T chamber controlled by a thermal instrument (Bioptechs, USA). The bottom of the chamber is made of an acoustically transparent polyimide film (Kapton Type HN, DuPont, USA) for minimizing a strong reflection from the below at the cell–chamber interface otherwise. Please note that the reflection coefficient is 0.94 at a water–stainless steel interface [13].

2.2. UM stimulation and confocal imaging systems

Fig. 1 illustrates the system layout including two sub-systems for UM stimulation and confocal fluorescence imaging. Conventional transducer fabrication procedure for acoustic microscopy [14] has been utilized to construct a single element ZnO transducer for 200 MHz UM generation. A 6 μm thick ZnO plate is sputtered between two gold electrode layers on the back side of a sapphire (Al_2O_3) buffer rod. UM arising from the ZnO plate is transmitted to a spherical cavity at the tip of the rod that works as a focusing lens. A quarter-wavelength glass layer is added to the front side of the cavity as an acoustic matching between the lens and the cell suspension.

The transducer's focal length is 546 μm and its aperture diameter is 570 μm. The transducer's bandwidth is 30% (Fig. 2a). It is practically impossible at present to determine the transmitted sound pressures from the transducer, because there are no established methods for calibrating absolute pressure level in UM's frequency range. The lateral beam profile is thus obtained by scanning a tungsten wire target that has a 3 μm diameter. The measured beamwidth is found to be 9.5 μm (Fig. 2b), close to the predicted value of 7.5 μm (= focal length × wavelength/aperture diameter) and the actual depth of focus is 60 μm (Fig. 2c). The excitation signal for the transducer is shown in Fig. 2d. 200 MHz sinusoidal bursts are supplied from a function generator (AFG 3251, Tektronix, USA) to the transducer, followed by a 50-dB power amplifier (525LA, ENI, USA). The resultant peak-to-peak (V_{pp}) voltage of the burst with the transducer being loaded is adjusted to 16, 32, and 47 V. The number of cycles is 5 per burst, and the *PRF* is 1 kHz.

Fluorescence live cell imaging is carried out to monitor the cellular response to UM stimuli with a Leica TCS SPE confocal microscope. Laser light chosen by an acousto-optical tunable filter (AOTF) is delivered to those cells for excitation (ex) after passing through an excitation pinhole, galvo-mirrors, a dichroic mirror, and a 40 × objective. The light emitted from the cells is then collected by the same objective and recorded in a photomultiplier tube (PMT) detector via an emission pinhole and AOTF, which allows the selection of proper emission (em) wavelengths.

2.3. Visualization of UM focus

For targeted cell stimulation, it is extremely important to accurately find the position of the UM's focal spot in the field of view (FOV) of interest, to ensure that 9.5 μm beamwidth is centered on individual cells whose mean diameter varies between 40 μm and 60 μm in this case. Temperature changes are probed to visually locate the UM's focus with Rhodamine B [15], a temperature-sensitive fluorophore. The transducer's position is controlled by a three-axis manual linear stage (OptoSigma, USA). The center of the transducer is first placed at the center of the FOV through bright-field imaging. Pulse echo tests are then conducted to axially fix the transducer at the UM's focal distance, the bottom surface of a delta T chamber. After a pulser (Model 5900PR, Panametrics, USA) with 200 MHz bandwidth drives the transducer only to transmit a 5 ns pulse to a flat quartz reflector, an echo is received