Master's Thesis

석사 학위논문

A novel ultrasonic stimulation system to enhance cell viability using piezoelectric micromachined ultrasonic transducers (pMUTs) with transwells

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A novel ultrasonic stimulation system to enhance cell viability using piezoelectric micromachined ultrasonic transducers (pMUTs) with transwells

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A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Robotics Engineering. The study was conducted in accordance with Code of Research Ethics[1]

01. 09. 2015

Approved by

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Professor Ji-Wong Choi (Signature) (Co-Advisor)

[1] Declaration of Ethical Conduct in Research: I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

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Accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

A new ultrasonic cell stimulation system was designed and fabricated using 10 by 10 and 10 by 29 piezoelectric micromachined ultrasonic transducer (pMUT) arrays. The sizes of pMUT arrays are 2.5 by 2.5 mm and 2.27 by 6.84 mm. The diameter and pitch of pMUT arrays are 120 μm and the measured resonance frequency of each single pMUT element was 1.494 MHz in water. The acoustic peak pressures are 0.22 MPa and 0.18 MPa for 10 by 10 pMUT and 10 by 29 pMUT, respectively. The spatial average temporal average (SATA) intensity are 612.8 ± 183 mW/cm^2 and 200 ± 22 mW/cm^2 which are converted by the acoustic pressure of 10 by 10 and 10 by 29 pMUT array.

The fabricated pMUT arrays were attached on print circuit boards (PCBs) and wire bonding was done for electrical connection. Each PCB and pMUT array was coated with parylene C for biocompatibility and waterproofing. For cell culturing, a twelve-well transwell was assembled one a PCB with ten pMUT arrays by epoxy. After curing the epoxy, polydimethylsiloxane (PDMS) was coated on the epoxy to enhance biocompatibility of the ultrasonic cell stimulation system. The size of the cell stimulation system is 13.4 X 17.3 X 2.5 cm and it is small enough to place the whole system in an incubator.

The fabricated ultrasonic cell stimulation system was used for cell stimulation to characterize the effect of ultrasonic cell stimulation for proliferation of PC12 cells. Ultrasonic cell stimulations were performed for 16 groups in combination of stimulation time for 5 min, 10 min, 20 min, and 30 min and duty cycle for 15 %, 30 %, 50 % and 70 %. There was also a control group without ultrasonic stimulation. All of the stimulated

groups showed positive effect on cell proliferation. For the most significant effect, the 5 min stimulation group showed more increased number of the cells as 238 %. Furthermore, the proliferation pattern was different along the stimulation time, not duty cycle. As a result, short stimulation time and long stimulation time occurred different tendency. Short stimulation groups showed 118 %, 122 %, and 134 % increase rate of cell number during stimulation, however long stimulation groups showed 117 %, 105 %, and 152 % increase rate of cell number during stimulation. Long time stimulation group has different increase pattern after last stimulation.

In this thesis, new ultrasound cell stimulation system using the pMUT was developed for overcoming the limitation of conventional cell stimulation system using bulk ceramic transducer. After cell stimulation for PC12 cells, all stimulation group has averagely 150 % increase rate of cell number than control group.

Keywords: Piezoelectric micromachined ultrasonic transducer (pMUT), Cell stimulation system, 12-well transwell, PC 12 cell, Proliferation.

Contents

List of Figures

List of Tables

1. INTRODUCTION

1.1 Background

In the world, many people has neural disease because of eating the western food, suffering from stress and less exercise. Neural disease from several disease that are brain stroke, aging, heart disease and etc. It can lead the death and aftermath. However, exact treatment method for healing those disease is still not developed. Thus many researcher have been studying for treatment of the neural disease. Especially, a neural cell stimulation is the prerequisite research for brain and various nerve disease. Cell stimulation research have been proceeding by many researcher groups, because this study is a fundamental in a treatment research area. It also have been studying by many researchers for rehabilitation and neural recovery treatment [1-5]. The several cell stimulation research for expedition of the cell differentiation and proliferation have been proceeding for finding cell mechanism of viability, differentiation and proliferation by stimulation. For this reason, several stimulation methods was used for cell stimulation, such as the electric[6], magnetic[7] and optic[8] method. The electric stimulation used 1 to 10 voltage with pulse. After electric stimulation, the cell was more differentiate than non-stimulation group. The magnetic cell stimulation used the peak intensity of the magnetic field with 34 mT. In the cell stimulation using the optic method, it used laser of 532 nm and 473 nm with 2 Jcm^{-2}. The optic stimulation also, helps to proliferation of the cell. However, these stimulation methods have limitation like an electric stimulation cause the damage to the cell[9], a magnetic stimulation has low resolution[10], and an optic stimulation has high attenuation[11].

1.2 Necessity of cell stimulation using the ultrasound

Ultrasound is an acoustic pressure which has frequency range above 20 kHz. We call ‘ultra’ because it is above human audible sound wave. Since ultrasound is just a kind of acoustic sound wave it is not harmful to human. The piezoelectric effect converts the electrical energy to mechanical energy or vice versa. Those effect was property of piezoelectric materials. These effect was first found in quartz and tourmaline crystals by Pierre and Jacques Curie brother in 1880. At normal quartz, dipole was not aligned. When the electrical field is applied, dipole was aligned along electric field direction. At that time, quartz can deform and it is called as piezoelectric effect. The other way, generated forces from the deformable quartz makes electric energy and it also piezoelectric effect. However, quartz and tourmaline crystal have low piezoelectric property. Thus, to obtain the high piezoelectric property, many researchers have been studying piezoelectric materials. As a results, lithium niobate (LiNb2O6), Barium titanate (BaTiO3), lead zirconate titanate (Pb(Zr,Ti)O3 or PZT) were developed. Those materials were used for sensors and actuators. For example, they can be used as transducer, artificial cochlear, energy harvester, accelerometer, micro actuator, sonar, range finder, flow sensor and bio-sensor.

Transducer was used for medical area. Because of the ultrasound has several advantage such as safety, simple device[12], real-time operation[13]. Especially, the ultrasound is safety than other treatment source such as radiation, electric, magnetic, optic and thermal, also it is possible to real-time operation. Thus, ultrasound have been used for diagnostic to pregnant. The ultrasound system had simple component that compose of transducer and generator. However, radiation system has complex and many component. To overcome those limitation, recently, it was reported that ultrasound can be used to stimulate cells to promote cell proliferation and dendrite growth[14, 15].

In cell research area, enhancing the good ability of neuronal cell is expected to expedite the recovery of damaged neurons. However, high intensity ultrasound damage to tissue or cell, because that cause the rising the temperature or make cavitation. Thus, high intensity ultrasound used for treat the tumor that system name is high intensity focused ultrasound (HIFU). However, in the cell stimulation, we stimulate and heal the cell, so low intensity ultrasound was used.

Low intensity pulsed ultrasound (LIPUS) have been used for cell stimulation and treatment. Recently. LIPUS is widely used for clinical area including physical therapy such as, bone fracture healing and drug delivery[16, 17]. Figure 1.1 was low intensity pulsed ultrasound effect to the bone healing of rabbits. LIPUS group was accelerated to bone healing than control group. Also, According to previous study, LIPUS has various advantages, such as promoted the growth of bacteria, increased sludge microbial movement and improved anaerobic fermentation process[18]. Furthermore it is used for cell stimulation for enhance the cell viability and promote proliferation and differentiation[14, 15].

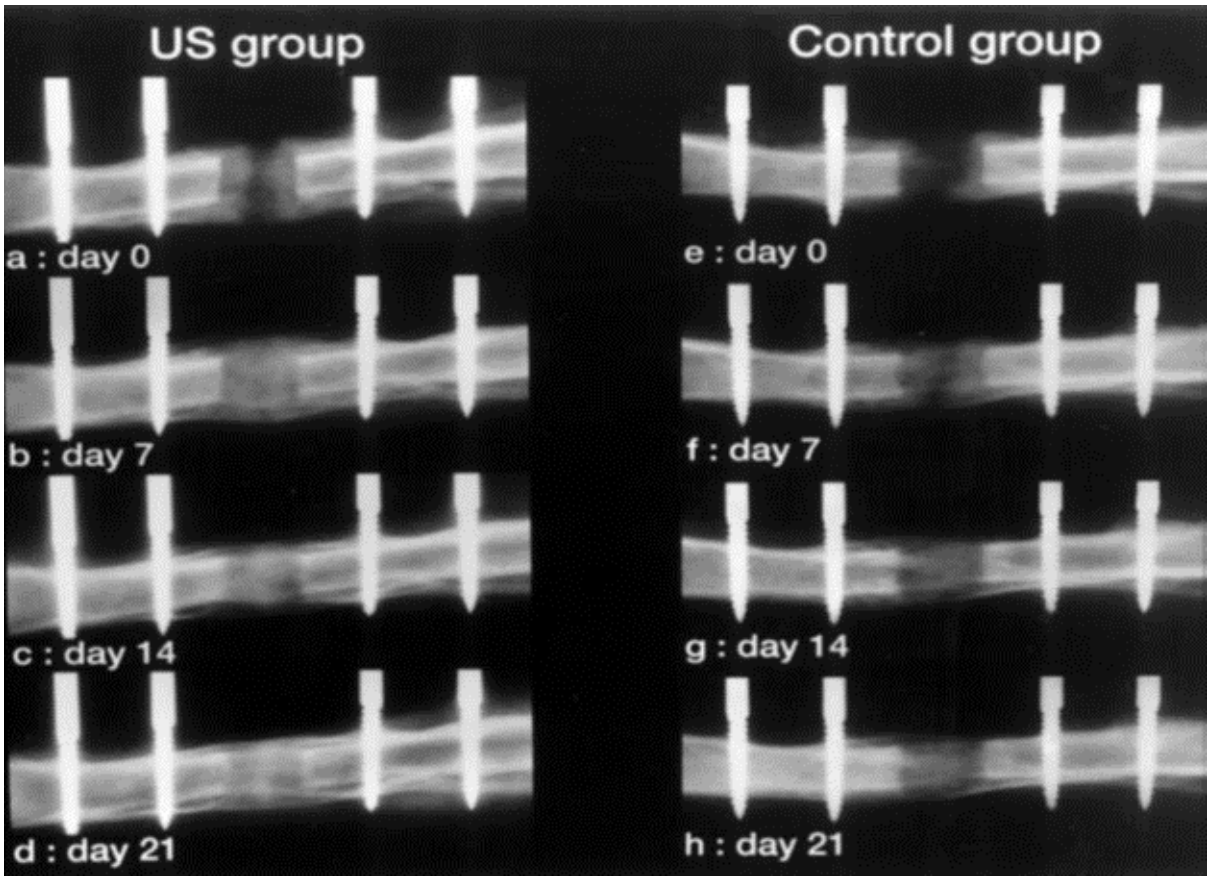


Figure 1.1 LIPUS accelerates bone healing in rabbits[17].

1.3 Research trend or Related research works

1.3.1 Cell stimulation system with bulk transducer

Normally, bulk ceramic transducer was used for cell stimulation. Bulk ceramic transducer, which make up the majority of transducer, lean to have a good effective coupling coefficient. Also, that used bulk ceramic piezoelectric material, which generate high intensity ultrasound. Thus, the traditional cell stimulation systems used transducer that composed of bulk ceramic. Figure 1.2 was shown for bulk ceramic transducer schematic view. However, these system has limitation. According to λ/2 pitch rule for designing transducer array, above 10 MHz frequency is hard to dice each element in bulk ceramic and expensive process where λ mention to the wavelength[19]. Also, traditional cell stimulation systems using the ultrasound are bulky, because these system used bulk ceramic transducer and it is not easy to characterize ultrasound intensity with the bulky stimulation system. Those systems also have limitations to study multiple cell stimulation parameters in a confined cell culture environment in real time. Because of conventional cell stimulation system is too bulky to enter the incubator[20-23].

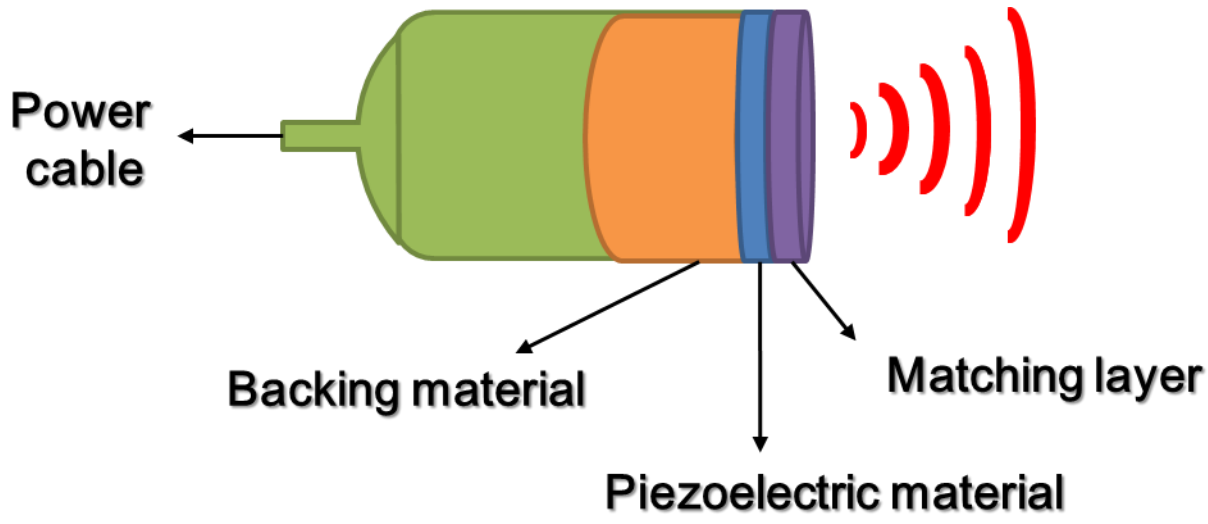


Figure 1.2 Bulk ceramic transducer schematic view.

1.3.2 Capacitive micromachined ultrasonic transducer (cMUT)

Capacitive micromachined ultrasonic transducers (cMUT) are widely considered as a bright alternative to the piezoelectric transducers. Wide research on the fabrication and modeling of cMUT began in the early 1990s [24-28]. The working mechanism of cMUT is named electrostatic transduction as shown the figure 1.3 [25]. It can be described by means of a parallel-plate capacitor where one plate is kept stationary, and the other membrane is forced in flexural vibration by a time varying voltage or by the reflection of the wave at its surface. The capacitance modulation due to membrane displacement is used to detect the signal. This basic principle of actuation and detection is not a new invention. However, it has not gained popularity simply because electric field strengths caused by the applied voltage are required to be on the order of million volts per centimeter, in order to achieve electrostatic forces as large as a kilogram per square centimeter [29]. It need high bias voltage, so it is not adopted to bio application. Applied high voltage over to pull in voltage, cMUT was not working. Also, cMUT fabrication process is difficult and complex. For those reason, cMUT still not used for cell stimulation.

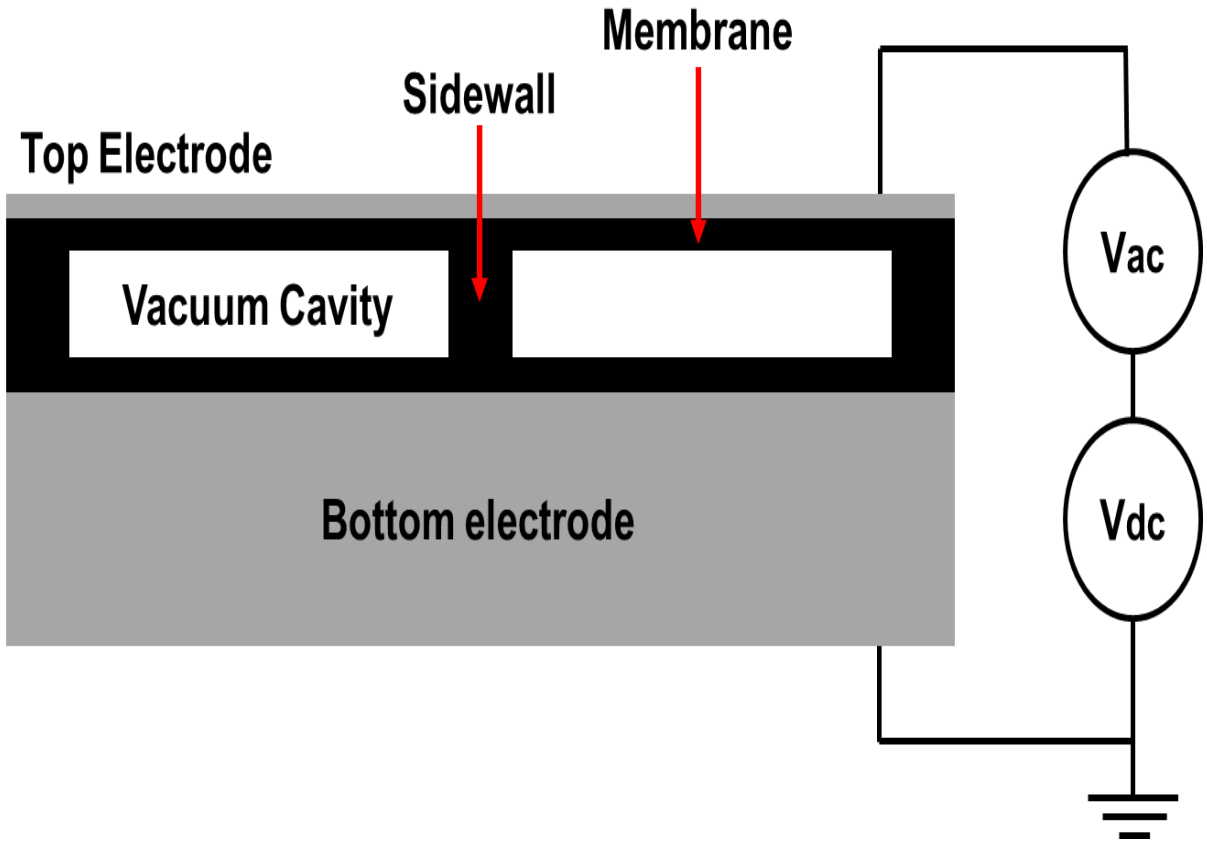


Figure 1.3 Capacitive micromachined ultrasonic transducers (cMUT) schematic view.

1.4 Objective of research

New cell stimulation system was composed of pMUT array and was designed to overcome the limitation of conventional cell stimulation system. The pMUTs made by micro-electro-mechanical-system (MEMS) technology and it is expected to offer several advantages over the traditional bulk piezoelectric transducers and capacitive micromachined ultrasonic transducer.

Figure 1.4 shows the cross-sectional view of a single element pMUT. The resonant frequency of bulk ceramic transducer is dependent on thickness of piezoelectric material, however the resonant frequency of pMUT is dependent on area and thickness of membrane. The pMUT has several advantages over conventional bulk ceramic transducer. A 2-D pMUT array requires much less wires during fabrication compared with conventional transducer, also, size is smaller than conventional transducer. Since those advantages make pMUT one of transducers to replace the bulk piezoelectric transducer, several groups have been researching to increase the performance of pMUT[31-37].

Thus, we suggest the new cell stimulation system composed of two dimensional (2D) pMUT array that using top cross over to bottom (TCTB) process technic and 12-well transwell. The pMUT array was made by TCTB technic, which not only solved wiring problem but also, possible to specific activate[35]. It is also possible to conduct experiment with several parameters at a time using a single pMUT array. Thus, it is expected to reduce the experimental cost and time consuming.

Therefore, pMUT can be used for cell stimulation in incubator and minimize the effect of external environment, so it is adopted to develop a novel ultrasound cell stimulation system. Furthermore the transducer was located under the petri-dish in conventional ultrasound cell stimulation system. This method cause attenuation of ultrasound intensity, so,

it can’t stimulate with accurate intensity[38]. Thus, the bottom of 12-well transwell was removed, ultrasound can be propagate to the cell without attenuation in this system.

This research used PC12 cells that is derived at adrenal medulla of rat. This cell have been widely used for model of neural proliferation and differentiation. Thus, many researchers have been used to get information of neural diseases. PC12 cells can possible to differentiation and proliferation. In this paper, check the three effects that is promoting proliferation of PC12 cells by ultrasound.

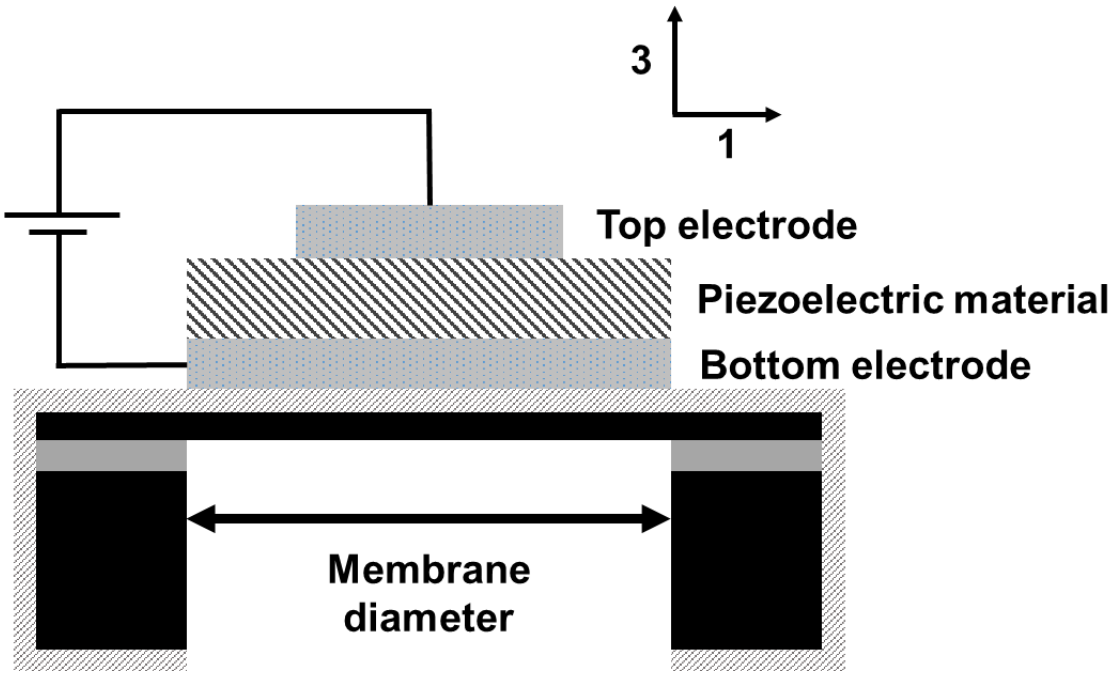


Figure 1.4 piezoelectric micromachined ultrasonic transducers (pMUT) working mechanism.

2. DESIGN AND FABRICATIONS

2.1 Cell stimulation system design.

The conventional cell stimulation system generating an ultrasound used bulk ceramic transducer. However, that system is expensive and size is bulky, so it has difficult to enter the incubator. To overcome those limitation, I did design the new cell stimulation system that composed of 2-D pMUT arrays and 12-well transwell. Figure 2.1 was shown the proposed schematic view of new cell stimulation system. This system is expecting a reduce the cell stimulation time and cost.

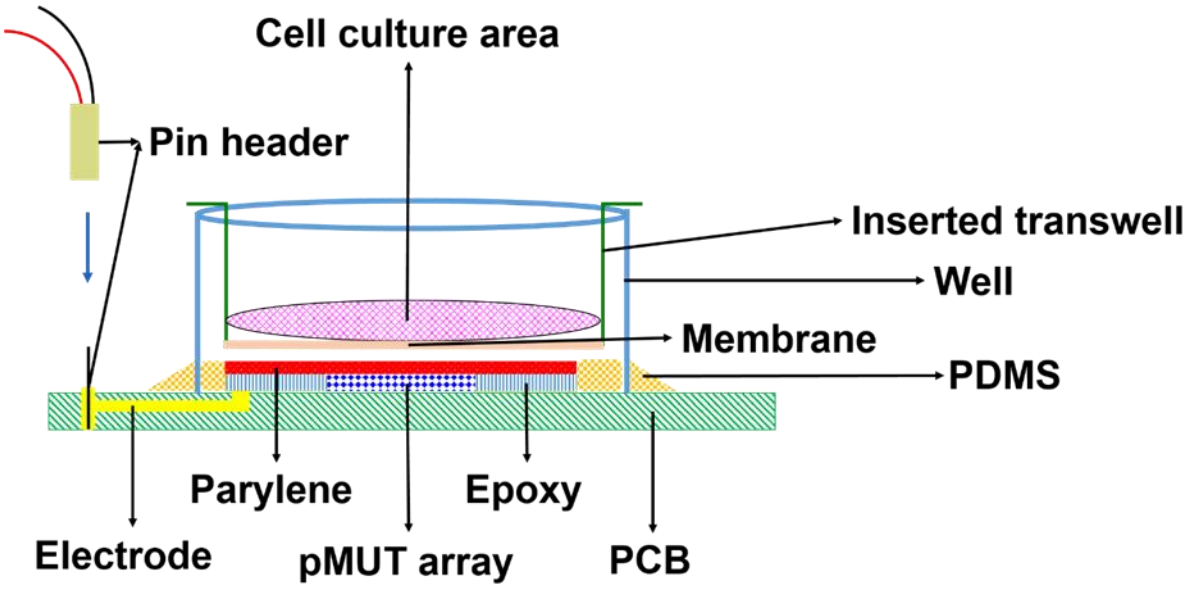


Figure 2.1 Concept image of cell stimulation system using the 2-D pMUT array and 12-well transwell.

A 2-D pMUT array was design by top-crossover to bottom (TCTB) technic that can be activate to want element in 2-D pMUT array. Each single pMUT fabricated circular K_{31} type pMUT. Figure 1.4 was shown for schematic view of the pMUT devices. A subscripts of K_{31} means the electric field or electric displacement direction and the stress or strain direction in the piezoelectric material layer, respectively. When an electric field is applied to the 3 direction in piezoelectric material, it generates strain in a 1 direction. An alternating current with frequency generate the strain of piezoelectric material in the membrane of the pMUT. The membrane was vibrated by given the frequency. Furthermore, when an acoustic pressure push the membrane of the pMUT, piezoelectric material generate the electric signal.

The resonant frequency of the pMUT is most important design factor. Because resonant frequency determine the application of the fabricated pMUT, such as 500 kHz to 2 MHz was used to therapy or stimulation, 1 to 10 MHz was used for imaging for diagnosis. In the pMUT, diameter of the pMUT was most important factor to determine the resonant frequency. The resonant frequency of circular membrane type pMUT is determined by the following equation:

$$f_0 = 0.47 \frac{t}{a^2} \sqrt{\frac{E_{avg}}{\rho_{avg}}} \quad \textbf{(2-1)}$$

Where t and a are the membrane thickness and radius, and E_{avg} and ρ_{avg} are the equivalent Young's modulus and equivalent laminated plate density[39]. The equivalent Young's modulus and density are calculated by following formulas:

$$E_{avg} = \sum_{k=1}^{n} \frac{E_k t_k}{t_{total}} \quad \textbf{(2-2)}$$

$$\rho_{avg} = \sum_{k=1}^{n} \frac{\rho_k t_k}{t_{total}} \quad \textbf{(2-3)}$$

In a table 1, the material properties and layer thickness used to calculate the resonant frequency. The resonant frequency calculated using Table 1 and 120 μm diameter of membrane. Designed pMUT has 1.5 MHz resonant frequency, which is suitable for cell stimulation[40, 41].

Materials	Young's modulus (Pa)	Density (kg m^{-3})	Thickness (μm)
PZT	6.30×10^{10}	7500	1.2
Pt	1.70×10^{11}	21440	0.38
SiO_2	7.50×10^{10}	2200	0.2
Si	1.25×10^{11}	2330	2.5
Ti	1.10×10^{11}	4510	0.2

Table 2.1 Material properties and layer thickness of pMUT, those are used for design the pMUT [33, 42].

2.2 Fabrication Process

2.2.1 Fabrication process for 2-D pMUT array

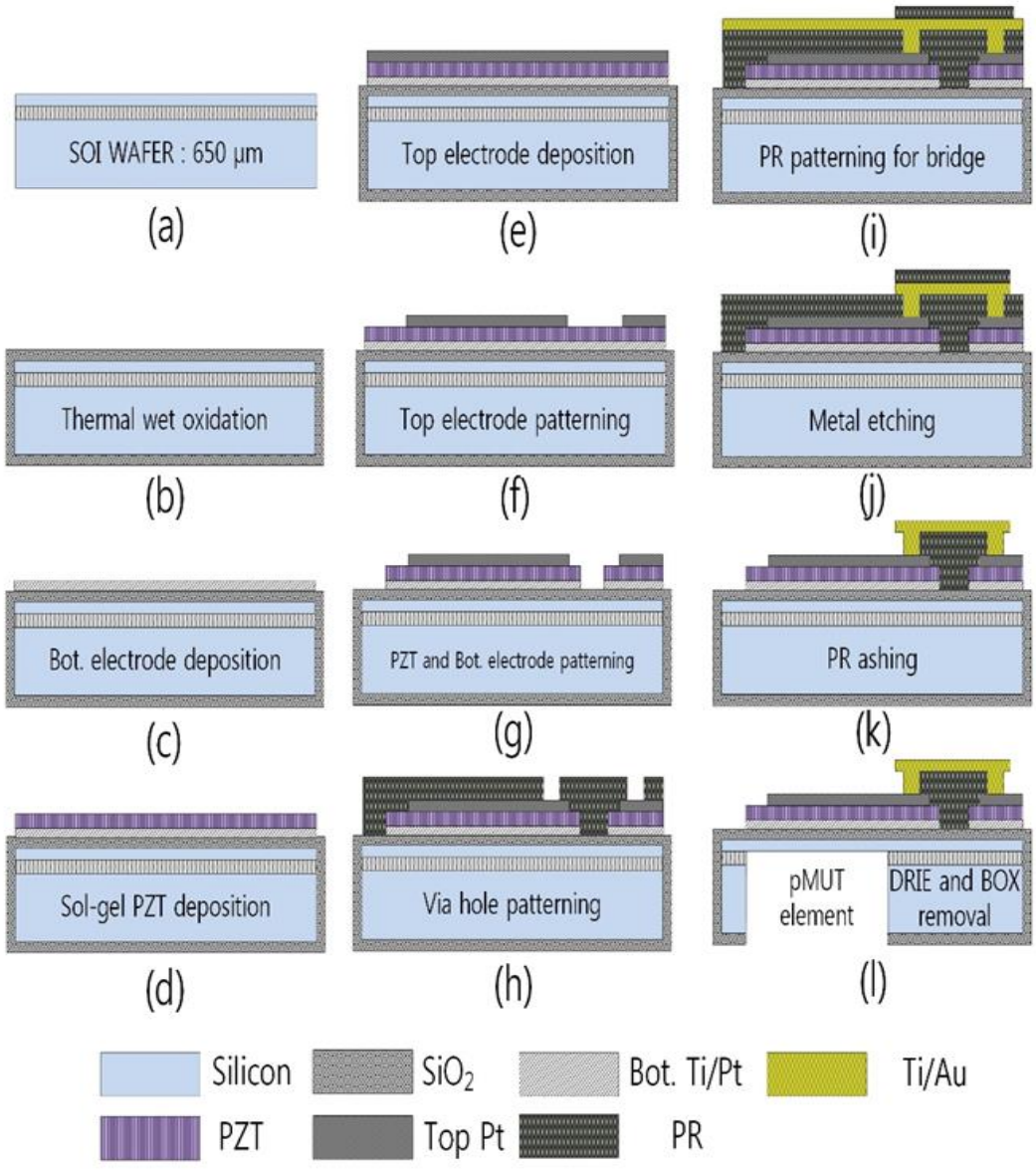


Figure 2.2 Fabrication process of 2D pMUT array with TCTB process technique: (a) prepared the 650 μm SOI wafer, (b) SiO2 was deposited by thermal wet oxidation, (c) Ti and Pt was sputtered for bottom electrode, (d) sol-gel PZT was spin coated for 1 μm, (e) Pt was sputtered for top electrode, (f) top electrode was patterned, (g) PZT and Bottom electrode was patterned by RIE process, (h) via hole was patterned for metal bridge, (i) Ti and Au was deposited and patterned, (j) metal was etched for metal bridge, (k) after metal etching, remained PR was removed by dry plasma washing process, (i) back side of the device was etched by DRIE process.

The fabrication flow chart for the 2D pMUT array is presented in figure 2.2. In this paper, TCTB process technique was used. The 2D pMUT array was fabricated on a duble-sided polished 6 inch silicon-on-insulator (SOI) wafer that was composed of a 2.5 µm thick device layer and 1.2 µm buried oxide layer under the device layer. The handle layer was located under the buried oxide layer with 550 µm thickness (figure 2.2(a)). The first fabrication process was wet thermal oxidation process with 200 nm which used insulator between device and substrate (figure 2.2(b)). 20 nm of Titanium and 180 nm platinum sputtered on oxide layer which was used for adhesive layer and bottom electrode. Furthermore, platinum (Pt) was served as a seed layer for sol-gel PZT growth (figure 2.2(c)). After bottom sputtered, the sol-gel PZT was coated on platinum layer with 1.2 µm. In this research, PZT thin film was composed of Pb1.10, Zr0.52 and Ti0.48 initial mixing ratio. The PZT thin film was coated by spin coating which was performed eleven times at a speed of 2500 rpm for 25 s. To show the piezoelectric characteristics, the thermal annealing process was needed. Thus, the spin coated PZT layer were thermally annealed for 10 min at 450 ℃ and 2min at 650 ℃ after each spin coating process for the first to fourth and sixth to ninth steps and fifth and tenth steps were annealed for 10 min at 650 ℃ after each spin coating steps. Finally, after last coating, PZT layer was annealed for 40 min at 650 ℃ (figure 2.2(d)). After PZT layer coating, a 200 nm thickness of Pt layer was sputtered on the PZT layer which was used top electrode (figure 2.2(e)). After top electrode sputtering, AZ 7220 photoresist (PR) and MIF300 developer was spin coated and PR was patterned by the photolithography. Patterned top electrode was dry etched using argon gas (Ar) and Chlorine gas (Cl_2) (figure 2.2(f)). The PZT layer and bottom electrode layer was patterned using the PR and developer. After patterned, PZT layer and bottom electrode was each dry etched using Boron trichloride (BCl_3) and Cl_2 gases and Ar and Cl_2 gases (figure 2.2(g)). Especially, the PZT layer was annealed for 40 min at 650 ℃ when after dry etching. AZ 7220 PR was patterned on

patterned layer for making a metal bridge (figure 2.2(h))and 200 nm thickness gold (Au) was sputtered on patterned layer. PR was one more coated and patterned (figure 2.2(i)). The Au metal bridge was patterned by reactive ion etching (RIE) using Ar and Cl_2 gases (figure 2.2(j)). The remaining PR on the Au layer was removed by dry plasma washing process using O_2 gas at room temperature (figure 2.2(k)). The process of (h) to (k) was TCTB process which make a metal bridge, it can be reducing the wire connection and individually activation in the pMUT array. The device layer of the wafer was blocked by PR for passivation during the deep reactive ion etching (DRIE) process. The backside layer of the wafer was patterned using AZ 7220 PR and then 500 nm thickness aluminum (Al) was deposited using electron beam evaporation which was used for hard mask during the DRIE process. After Al deposition, Al was patterned by lift off process using PR stripper (EKC830). The backside of the wafer was dry etched by DRIE process that used sulfur hexafluoride (SF_6) and octafluorocyclobutane (C_4F_8). After DRIE process, remaining SiO_2 layer was removed by Ar and CF_4 mixed gases and the passivation PR on the device layer was removed using dry plasma washing process. Figure 2.3 shown the completed 10 by 29 2D pMUT arrays for cell stimulation with metal bridge.

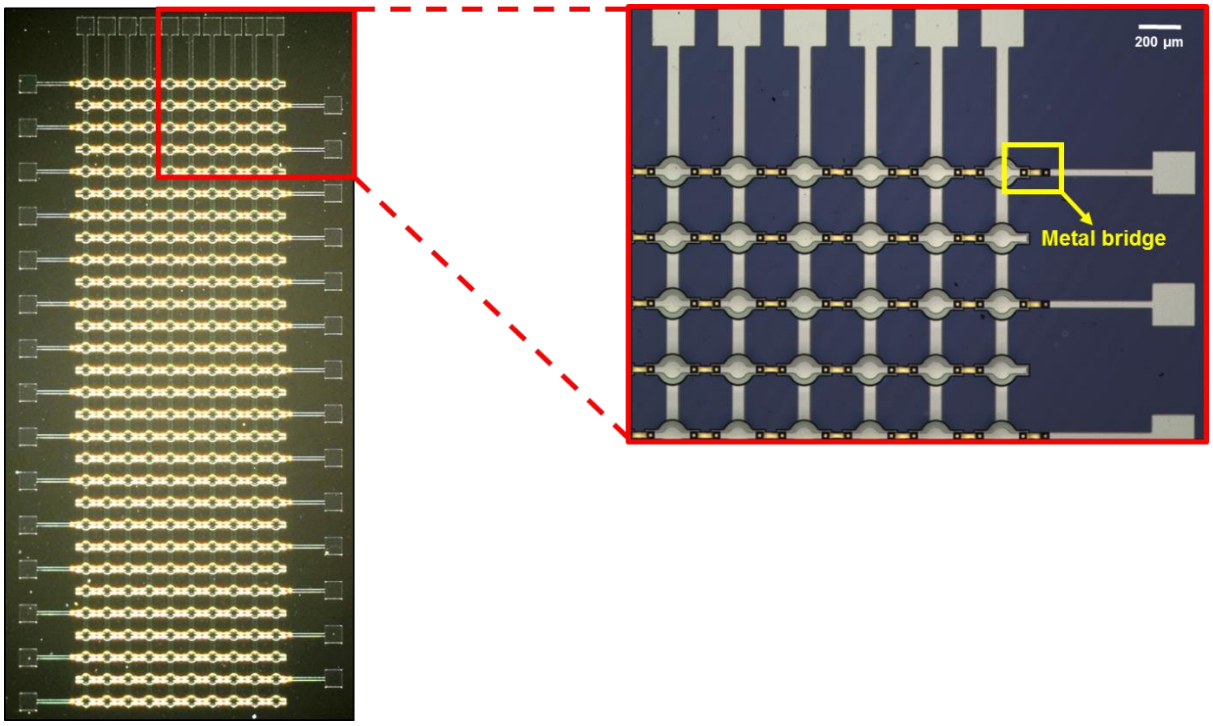


Figure 2.3 Fabricated 10 by 29 2D pMUT array using TCTB process technique.

2.2.2 Assemble the 2-D pMUT array and 12-well transwell

The 12-well transwell (3493, Corning Incorporated, USA) was used for cell culture which was composed of 12-well transwell, well cover and twelve inserted well. Diameter of inserted transwell is 12 mm, it also has membrane which has pore of 0.4 μm and cell was seeded on it. A membrane made by clear polyester and has special coating for good cell adhesive. The bottom of the well was removed for reduce the attenuation of intensity of ultrasound using the hand drill (Dremel 300, Dremel Robert Bosch Tool Corporation, USA).

The print circuit board (PCB) was designed according to the size of the 12-well transwell that size was 17.3 X 2.5 cm. Then, 10 by 10 and 10 by 29 of pMUT arrays were connected to PCB for electrical connection with wire. All of wire was protected by epoxy (figure2.4). After curing the epoxy, PCB board with bonded pMUT was coated with 2.55 μm parylene C for waterproofing, insulating and biocompatibility A thickness of coated parylene was measured using surface profiler (Dektak XT, Bruker, USA) (figure2.5). After parylene coating, PCB board bonded using the epoxy and Polydimethylsiloxane (PDMS) coated. PDMS consist of elastomer (Sylgard 184, silicone elastomer base) and cure (Sylgard 184 silicone elastomer curing agent). The elastomer and cure was mixed for 10 to 1 mixing ratio. After mixing, PDMS was curried on the epoxy with 12-well transwell that bottom was removed, in the oven for 1 hour, at 80 °C. The reason of used the PDMS, it is more the biocompatibility than commercial bond or epoxy, so it need not extra coating process. Figure 2.6 shown the completed cell stimulation using the 2D pMUT array and 12-well transwell.

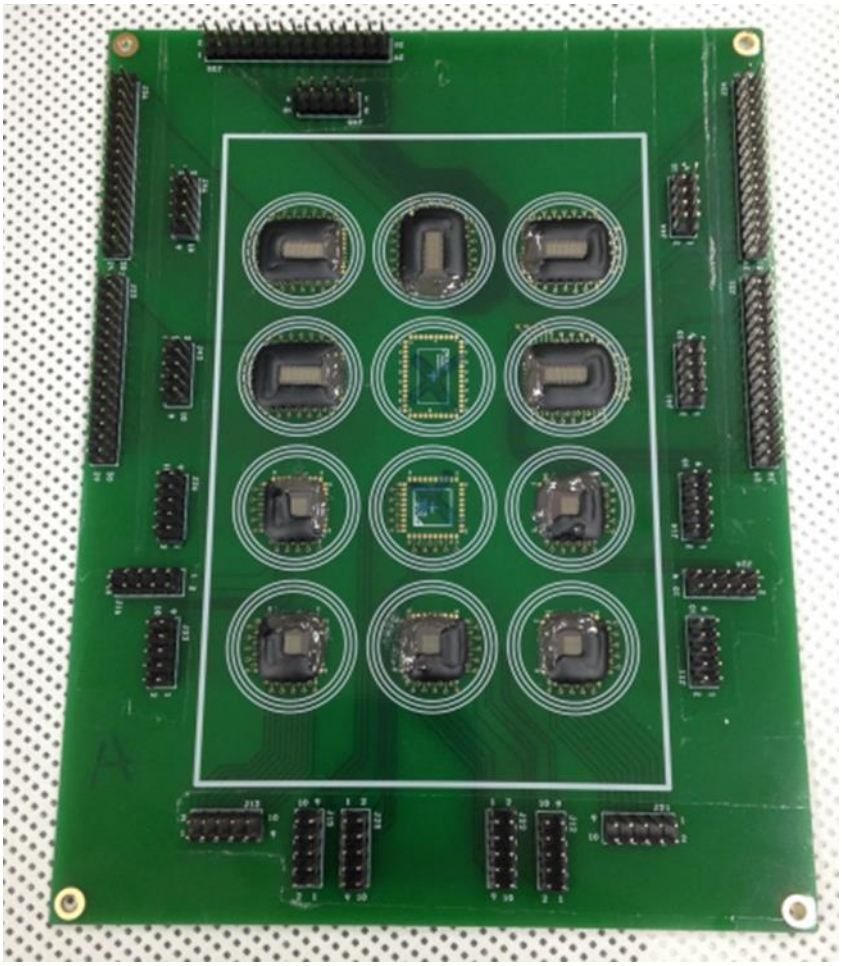


Figure 2.4 the pMUT array was bonded on the PCB, designed to fit on 12-well transwell.

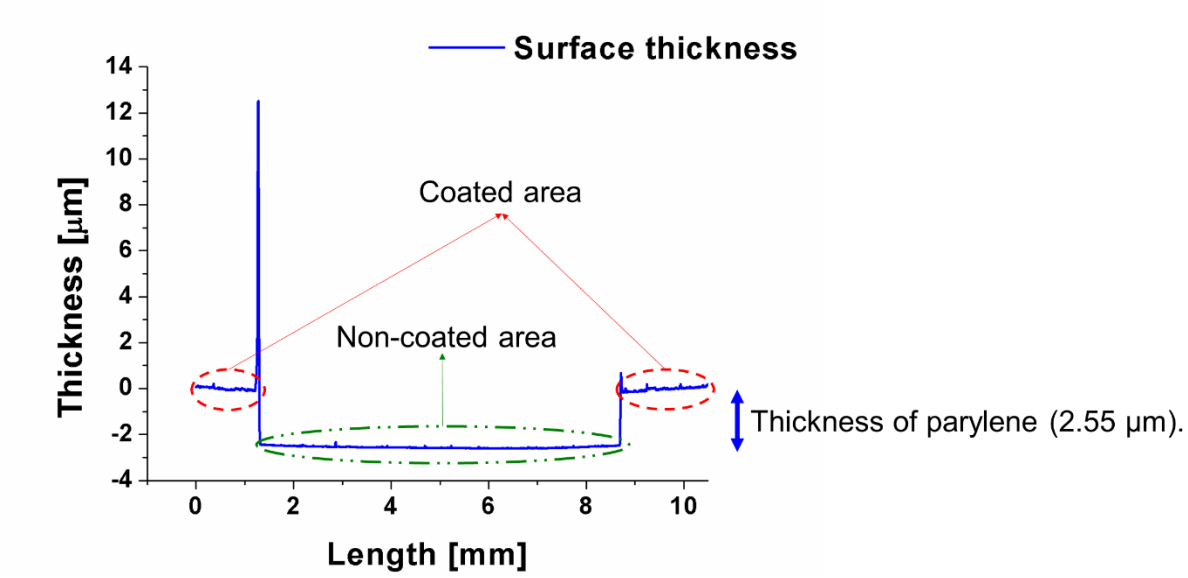


Figure 2.5 After parylene coating, thickness of the PCB surface was measured by surface profiler.

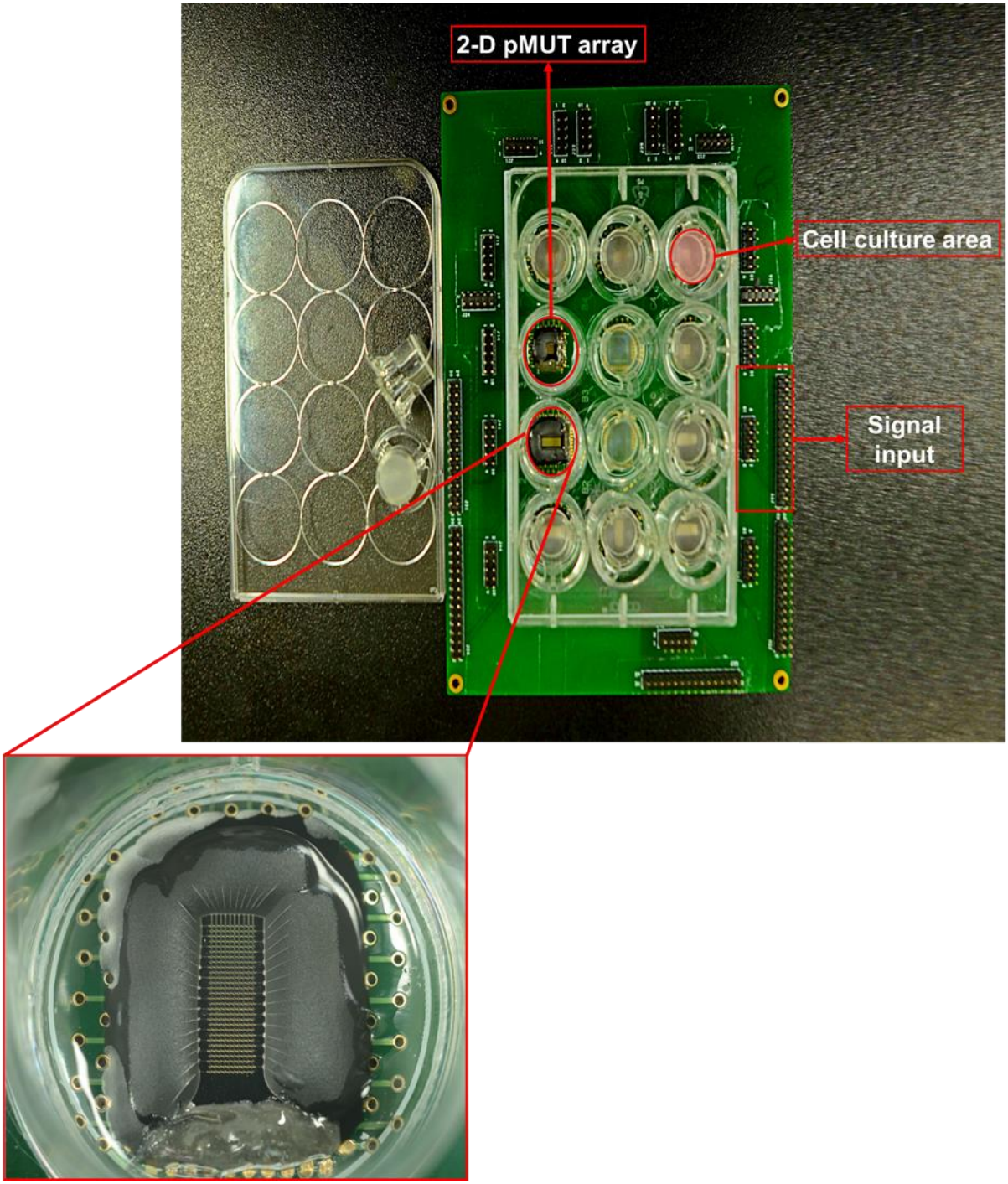


Figure 2.6 Fabricated ultrasonic cell stimulation system using pMUT array and 12-well transwell. Inset is an optical view of 10 by 29 pMUT array.

3. CHARACTERIZATION AND EXPEREIMENT SETUP

3.1 Impedance analyze of the 2D pMUT array

The fabricated pMUT has K31 type. Figure 1.4 is cross sectional schematic view of pMUT. This device working on 3 and 1 direction by applied AC voltage that included resonance frequency. Figure 3.1 was Butterworth-van Dyke equivalent circuit model, it was used for deriving characteristics of elements in the pMUT array. pMUT element of resonance frequency was measured by impedance analyzer (4294A, Agilent Technology, USA) in air. I choose and measured the 20 pMUT with 5 V dc voltage applied. The resonant frequency and anti-resonant frequency are related with coupling coefficient. The coupling coefficient is used for evaluating performance of the pMUT array. The coupling coefficient was obtained by following formula:

$$f_r = \frac{1}{2\pi\sqrt{L_1 C_1}} \quad \textbf{(3-1)}$$

$$f_a = \frac{1}{2\pi}\sqrt{\frac{C_0 + C_1}{L_1 C_0 C_1}} \quad \textbf{(3-2)}$$

$$k_{eff}^2 = \frac{C_1}{C_0 + C_1} = \frac{f_a^2 - f_r^2}{f_a^2} \quad \textbf{(3-3)}$$

Where C_0 shows the static capacitance of the pMUT element in the nonappearance of piezoelectricity. R_1, L_1 and C_1 represent the dynamic motional resistivity, inductance and capacitance, respectively, in the presence of piezoelectricity. In the Butterworth-van Dyke equivalent circuit model R_1, L_1 and C_1 is appropriate to values of mechanical damping, membrane stiffness and mass[43].

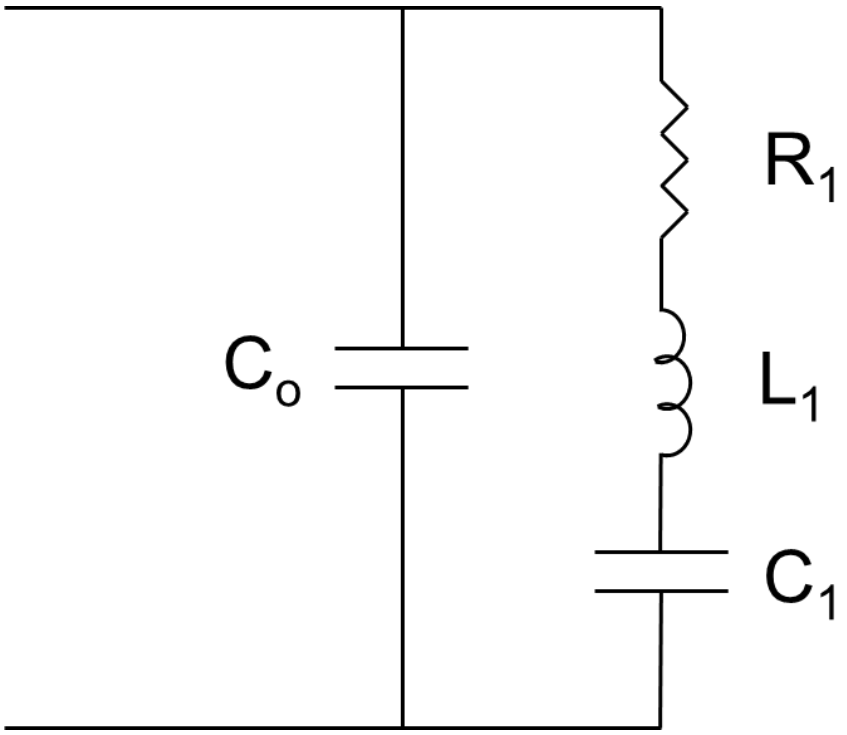


Figure 3.1 Butterworth-van Dyke equivalent circuit that is modeling for pMUT[43]

3.2 Intensity of the 2D pMUT array

An acoustic intensity is most important parameter in cell stimulation using ultrasound. The high intensity ultrasound used for kill the cancer cell it has 1000W/cm^2 of intensity. Also, 1 W/cm^2 and 2 W/cm^2 was used for physical therapy for ankle, elbow and wrist. In diagnostic area, 1 to 10 mW/cm^2 was used. Appropriated intensity of ultrasound promote the cell proliferation or differentiation it has 20 ~ 10000 mW/cm^2 intensity rage. Thus, intensity characterization is important in cell stimulation system. An acoustic intensity was measured by acoustic intensity measurement system (AIMS, Onda Corp., USA) (Figure 3.2). This measurement system was composed of water tank and X, Y, Z automatically moving stage system, hydrophone (HNC-1000, Onda Corp., USA) for convert acoustic pressure to electrical signal, and deionized (DI) water, function generator for input signal to pMUT, pre-amplifier for amplify output signal from the hydrophone and oscilloscope.

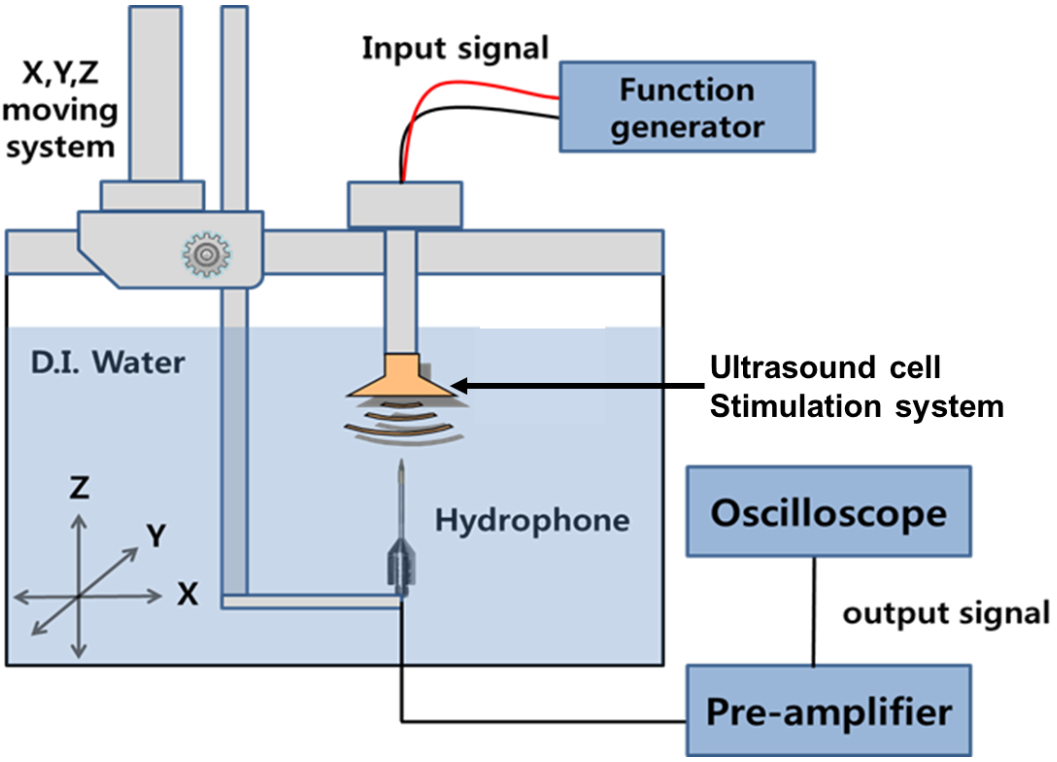


Figure 3.2 Schematic view of acoustic intensity measurement system with ultrasound cell stimulation system.

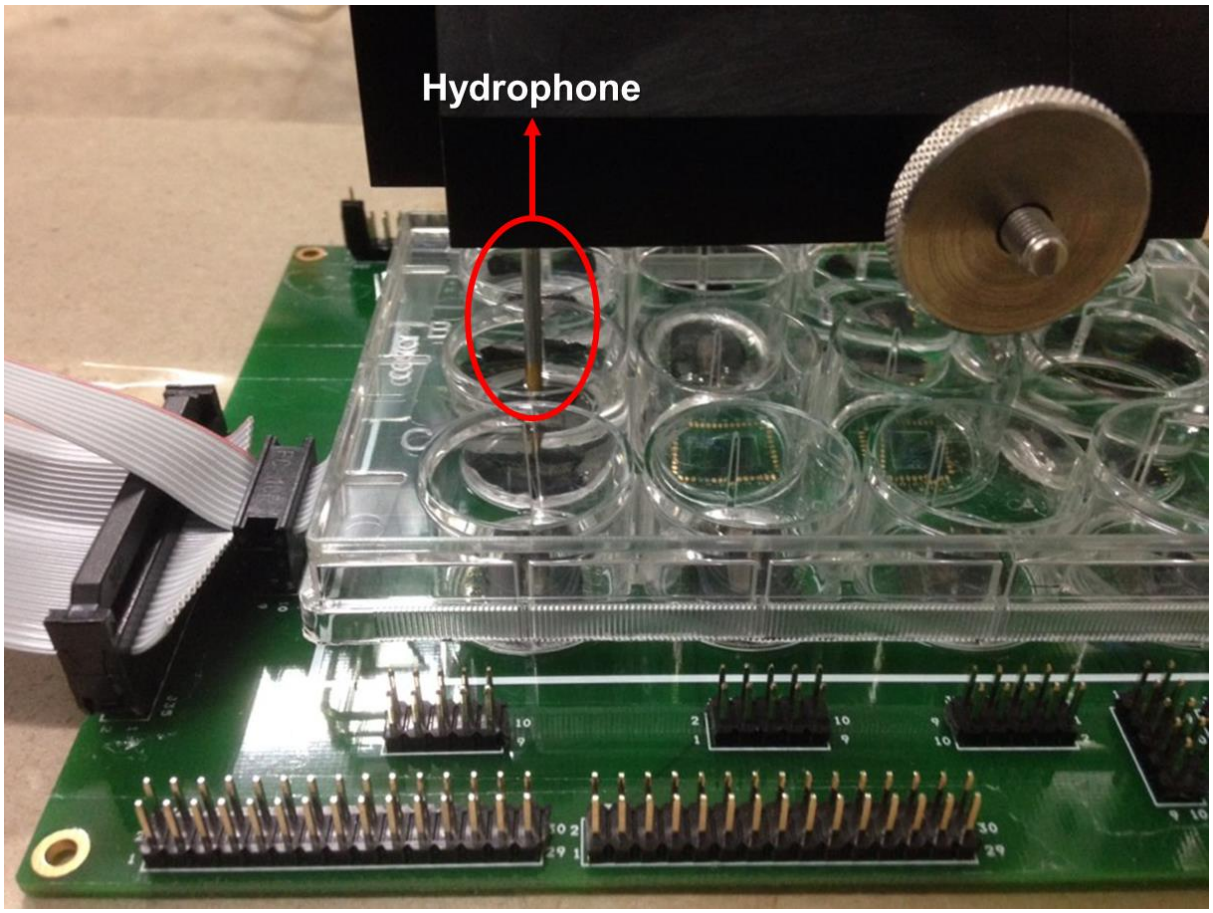


Figure 3.3 Closed view of ultrasonic intensity measurement system with a hydrophone placed 1 mm away from the pMUT array.

In this research, fabricated 10 by 10 pMUT array and 10 by 29 pMUT array was used to measure acoustic intensity. Figure 3.2 was shown the general method for acoustic intensity with transducer. The general method for measured acoustic intensity, hydrophone was located under the transducer. However in this research, hydrophone was located on the transducer, because electrical connection pin was not coated for waterproof as figure 3.3. Each pMUT array was applied by 5 V_{pp} AC voltage and 1.48 MHz ± 0.13 MHz of resonance frequency by function generator (33522B Waveform generator, Agilent Technology, USA). The hydrophone located 1 mm away from the pMUT in Z axis to measure acoustic pressure. Because the membrane of inserted well for cell culture is 1 mm away from the pMUT array.

3.3 Cell stimulation setup

3.3.1 Cell stimulation plan

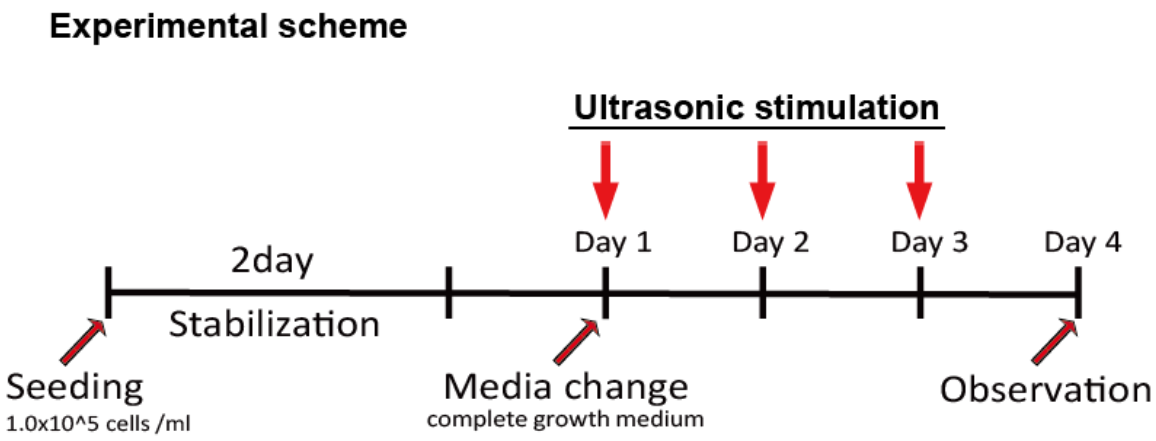


Figure 3.4 Cell stimulation plan with stimulation for three time.

In this research, PC12 cells were used for stimulation by new ultrasound cell stimulation system. PC12 cells were seeded on inserted membrane for 1.0 X 10^5 cells/ml. After seeding the cells, two day spent for stabilization. After stabilization, three day was used for stimulation to the cell, before the first stimulation media was changed to complete growth medium. In this research, ultrasound stimulation progressed with several parameter as stimulation time and duty cycle. The stimulation time and duty cycle each has four parameter, 5 min, 10 min, 20 min, and 30 min, 15 %, 30 %, 50 %, and 70 % duty cycle. The stimulation time and duty cycle was combined, so added control group and combined the 16 parameter was used for cell stimulation. After three time stimulation, all stimulated cells and control cells were observed using optical microscope and fluorescent microscope (Figure 3.4).

3.3.2 Cell culture

PC12 cells was chosen as the model for cell stimulation, this cell was cultured under the ordinary cell culture condition. PC12 cells (ATCC, USA) were maintained on inserted well coated by 0.01% poly-L-lysine (Sigma-Aldrich, USA) in Roswell Park Memorial Institute medium supplemented with 10% horse serum, 5% fetal bovine serum (Hyclone, Thermo Fisher Scientific, USA) and 1% antibiotics, at 37˚C in 5% CO2.

3.3.3 Determination of cell proliferation – Cell statistical analysis

Whole plate was divided into nine sections and every microscopic area was selected randomly by scanning from top to bottom. Each experiment was conducted in triplicate, and nine images were used to analyze each method. For optical image analysis, the number of cells was measured. Comparisons among different condition were done by the one-way ANOVA. Results are presented as mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered significant.

3.3.4 Determination of cell proliferation – immunocytochemistry

Cells were harvested at various conditions with ultrasonic stimulation. For harvesting, they were rinsed briefly with PBS and fixed with 4% (w/v) paraformaldehyde in PBS (4% PFA) for 30 min at room temperature. The cells were immersed in 0.5% Triton X-100, which contained Dulbecco's phosphate-buffered solution (DPBS) (Thermo, USA), at room temperature. For immunostaining, the cells were incubated with DPBS containing 4% normal donkey serum (NDS) (Jackson ImmunoResearch, USA) for 1 h, and exposed to diluted 1°C

antibody at appropriate concentrations (KI67, 1:500) in block solution overnight at 4℃. Subsequently, the cells were washed twice with DPBS and incubated with fluorescein isothiocyanate (FITC) conjugated antibodies (Jackson Immuno Research, USA), diluted at 1:1000 at room temperature for 2 h. We visualized the nucleus of cells cultured in the inserted using DAPI (Vector, USA) and β-actin under a vivatome microscope (Zeiss, Germany).

4. RESULTS AND DISCUSSIONS

4.1 Electrical characterization of the pMUT array

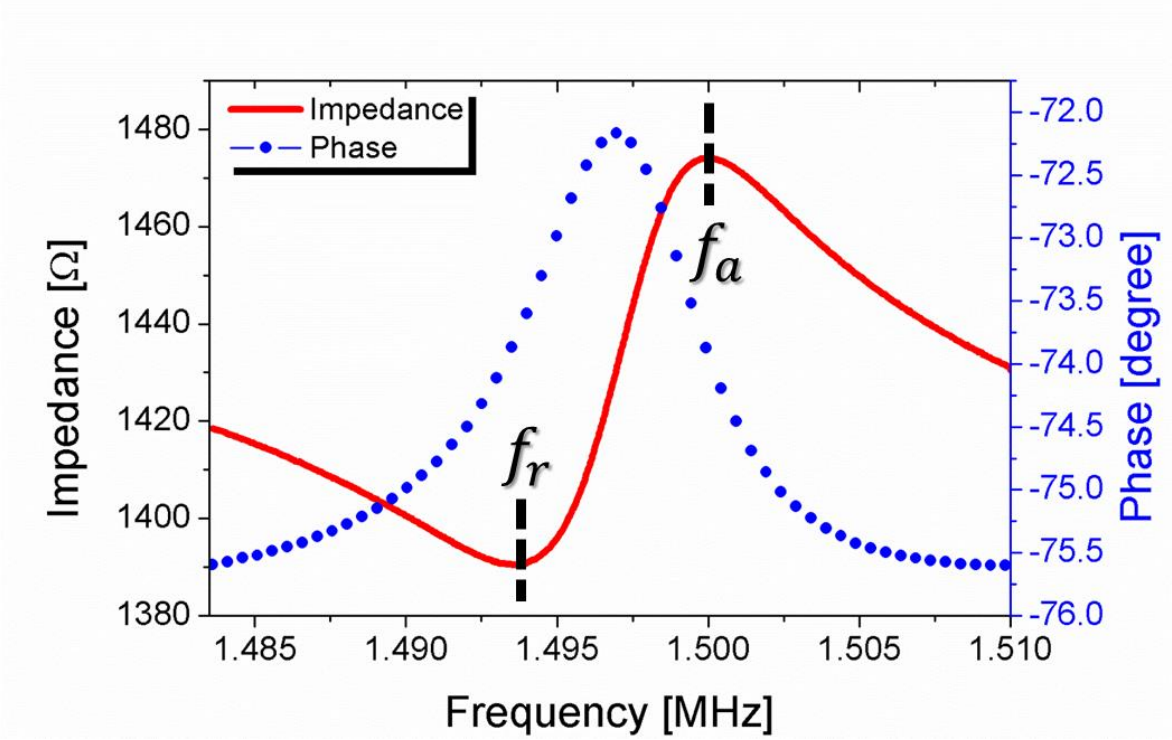


Figure 4.1 Impedance and phase measurement result of the pMUT array.

As shown in figure 4.1, resonance frequency and anti-resonance frequency of the pMUT. The resonant frequency was 1.493 MHz and anti-resonant frequency was 1.5 MHz. This resonant frequency rage was used for cell stimulation or physical therapy. In the fabricated pMUT array, those are component of the Butterworth-van Dyke equivalent circuit with $R_1 = 14\ k\Omega$, $L_1 = 0.33\ H$, $C_1 = 125\ fF$ and $C_0 = 149\ pF$. The average measured in air resonant frequency of 20 pMUT array was 1.41 MHz and coupling coefficient are 1.18 % with 5 V dc voltage applied. The coupling coefficient is conversion ratio between the input

electrical energy and the output mechanical energy, or vice versa. The effective coupling coefficient are determined by piezoelectric material and geometry of the pMUT element.

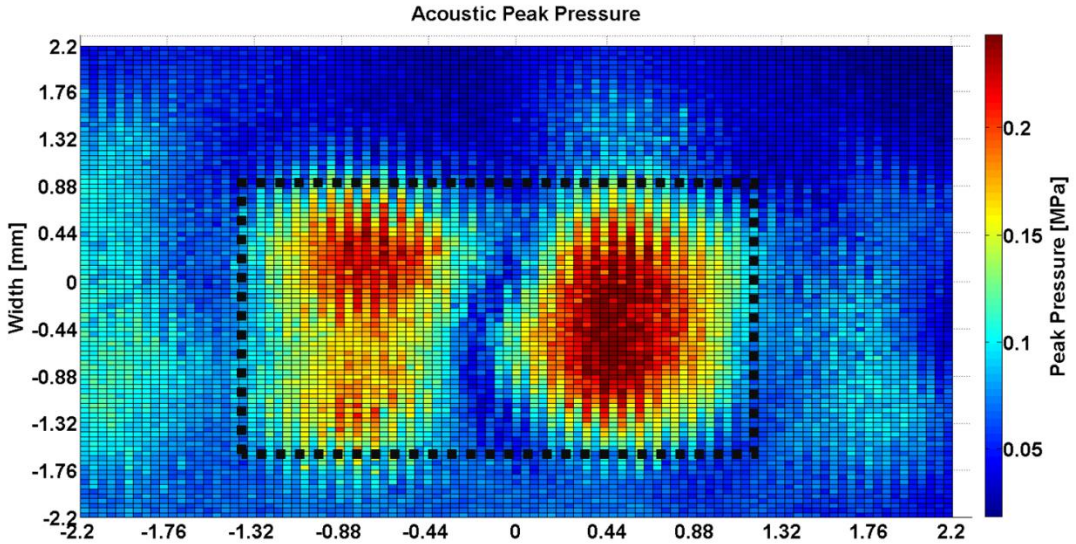
4.2 Acoustic characterization

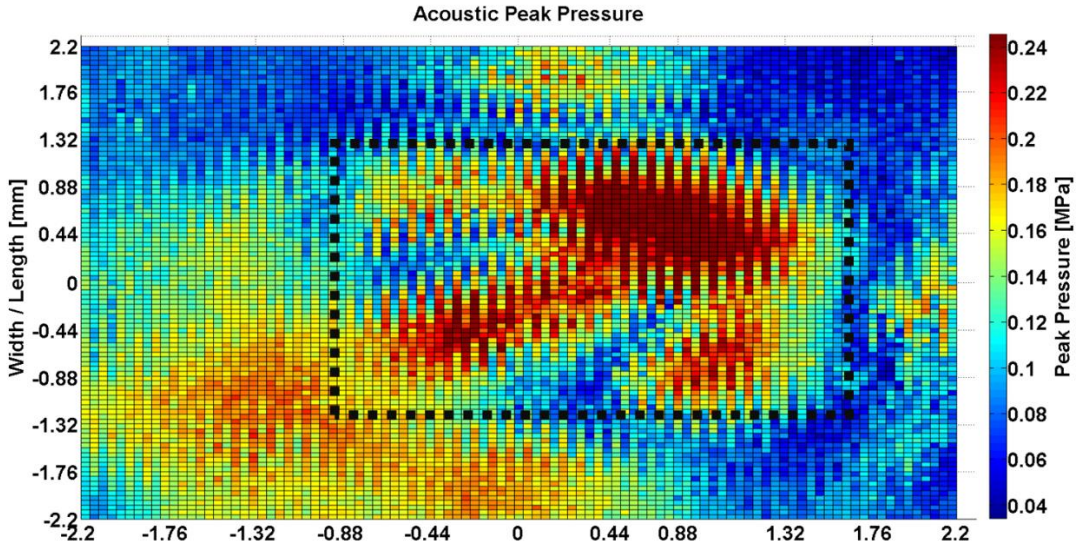
The results of the acoustic intensity of 10 by 10 pMUT array and 10 by 29 pMUT array was 0.25 MPa ± 0.002 MPa and 0.18 MPa ± 0.03 MPa acoustic pressure respectively. Figure 4.2 was shown for results of 10 by 10 pMUT array and figure 4.3 was shown for results of 10 by 29 pMUT array, also the black dot on the figure 4.2 and 4.3 that is indicating line for location of pMUT array. All of the pMUT array was applied 5 V_{pp} AC voltage with

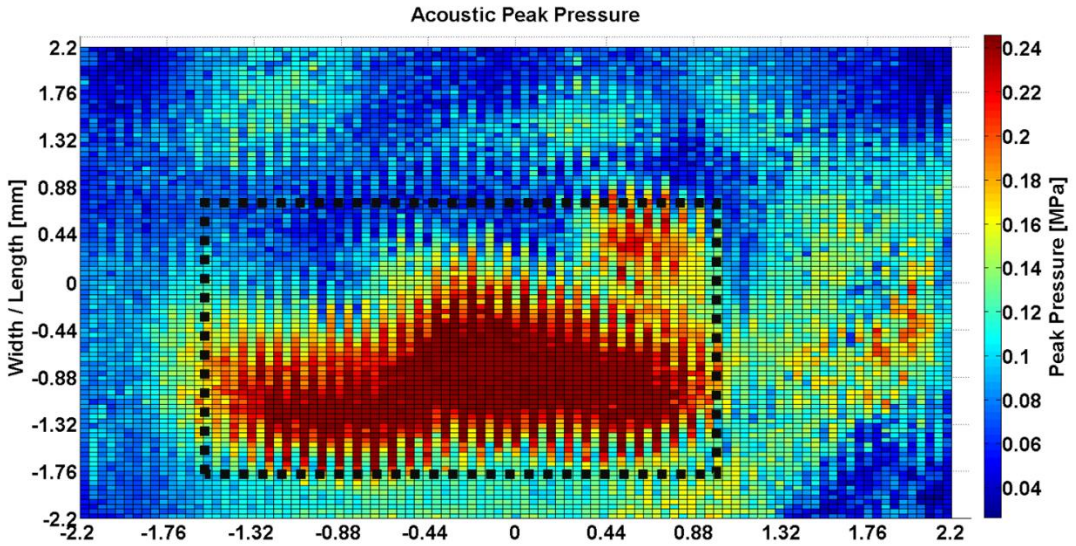
1.48 MHz ± 0.13 MHz of resonance frequencies. In the figure 4.3 four red line of results graph, those array was used for cell stimulation because the results of the acoustic pressure has uniformity acoustic pressure at the pMUT array. Also measured acoustic pressure was converted to acoustic intensity. The converted intensity was 200 ± 22 mW/cm^2 spatial average temporal average (SATA) intensity type which intensity range adopted for cell stimulation. Those SATA intensity value was appropriate converting value by acoustic pressure to acoustic intensity, because, according to Loreto B. Feril Jr et al. in Toyama medical and pharmaceutical university in Japan, they converted acoustic pressure to acoustic intensity that results are similar to my research. The table 4.1 was shown for converted results by Loreto B. Feril Jr group [44]. Thus, fabricated cell stimulation system using the pMUT array has intensity for appropriate for cell stimulation.

Reading Output (W/cm^2)	0.1	0.3	0.5	0.7	1.0
I_{SATA} at 10 % Duty Cycle (W/cm^2)	0.048	0.081	0.105	0.123	0.159
Acoustic Peak Pressure (MPa)	0.061	0.132	0.146	0.179	0.204

Table 4.1 I_{SATA} and peak acoustic pressure value. [44].


Acoustic Peak Pressure
Width [mm]
Peak Pressure [MPa]
2.2
1.76
1.32
0.88
0.44
0
-0.44
-0.88
-1.32
-1.76
-2.2
0.2
0.15
0.1
0.05


Acoustic Peak Pressure
Width / Length [mm]
Peak Pressure [MPa]
2.2
1.76
1.32
0.88
0.44
0
-0.44
-0.88
-1.32
-1.76
-2.2
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04


Acoustic Peak Pressure
Width / Length [mm]
Peak Pressure [MPa]
2.2
1.76
1.32
0.88
0.44
0
-0.44
-0.88
-1.32
-1.76
-2.2
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04

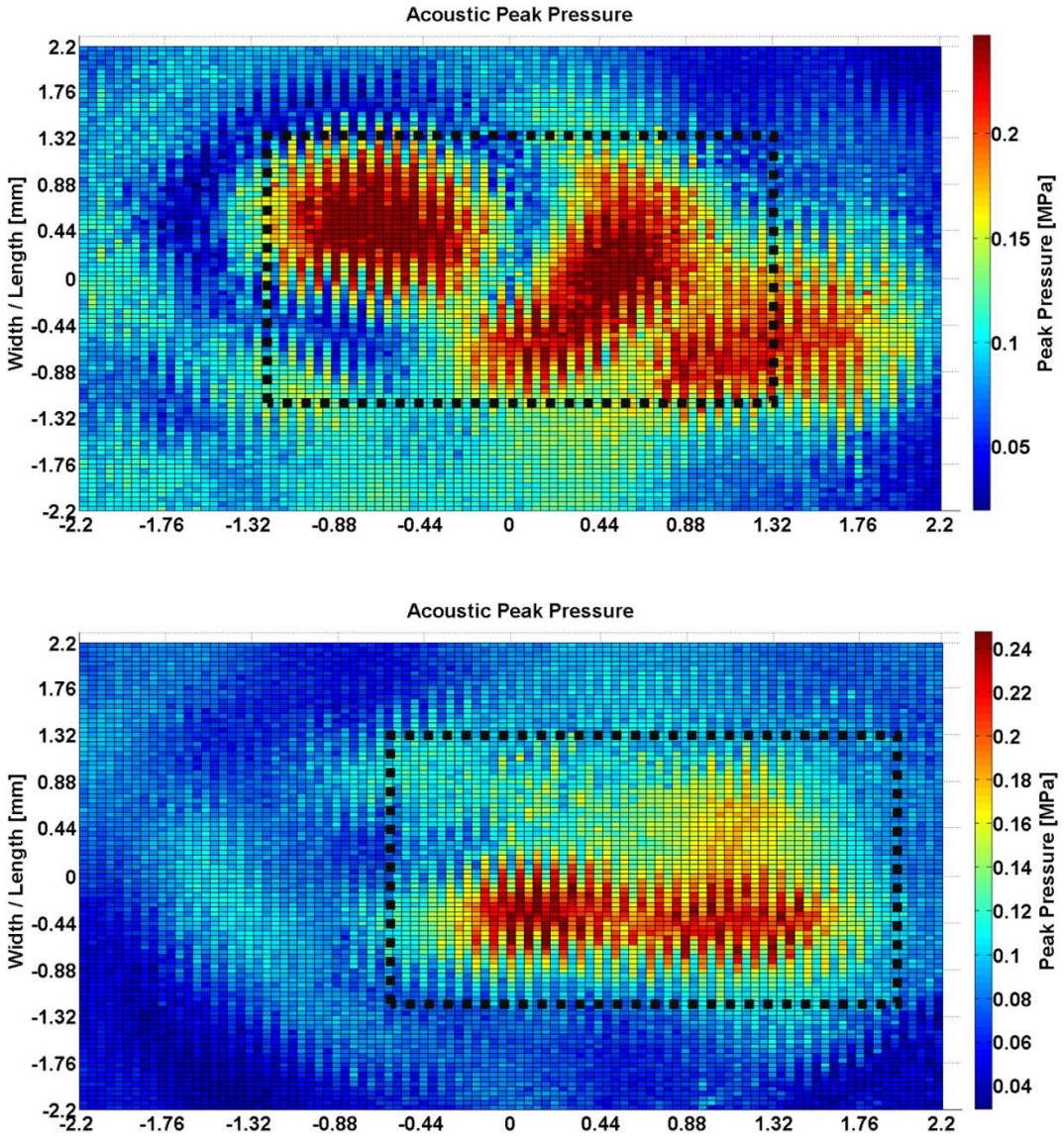
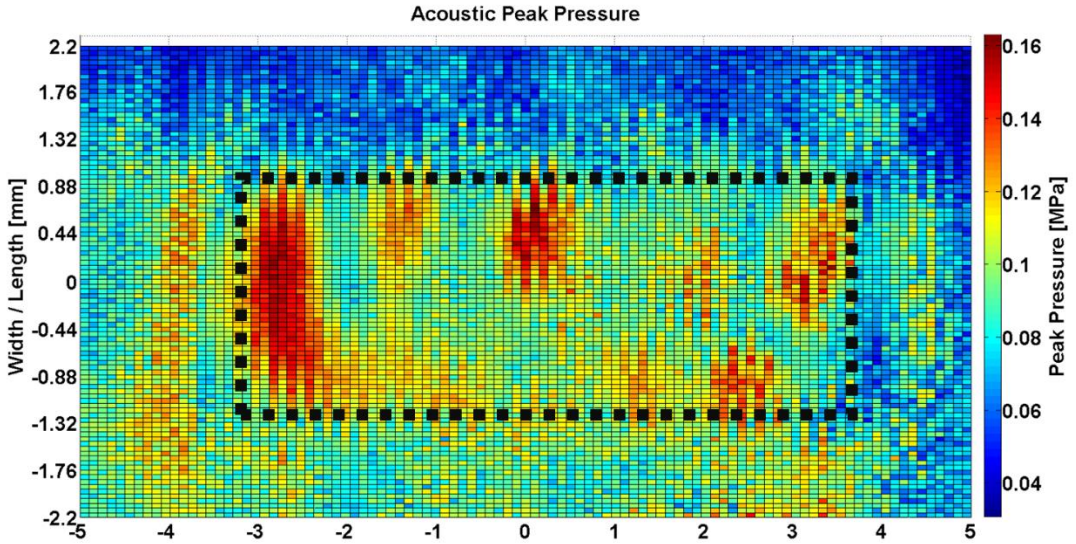
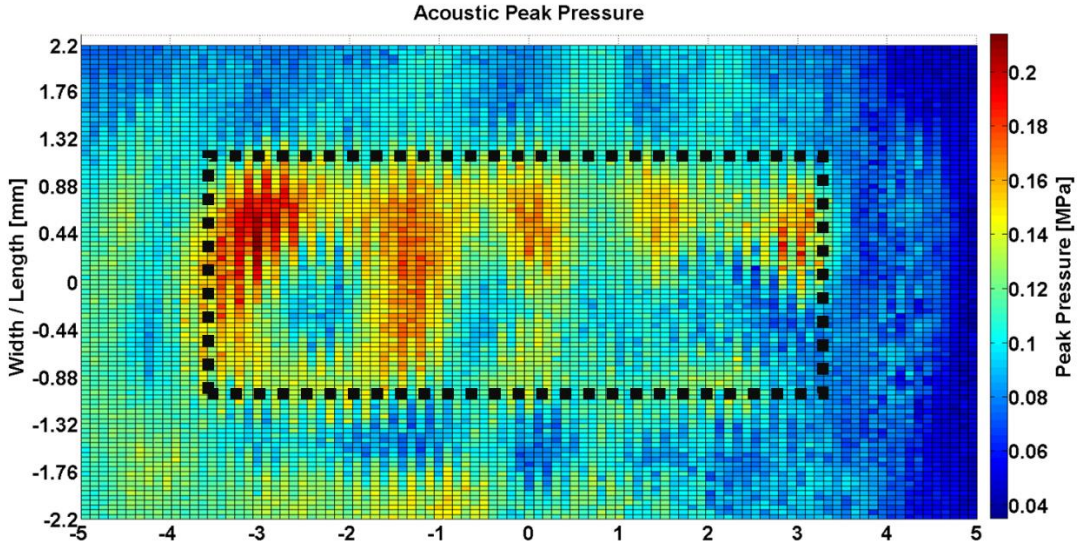
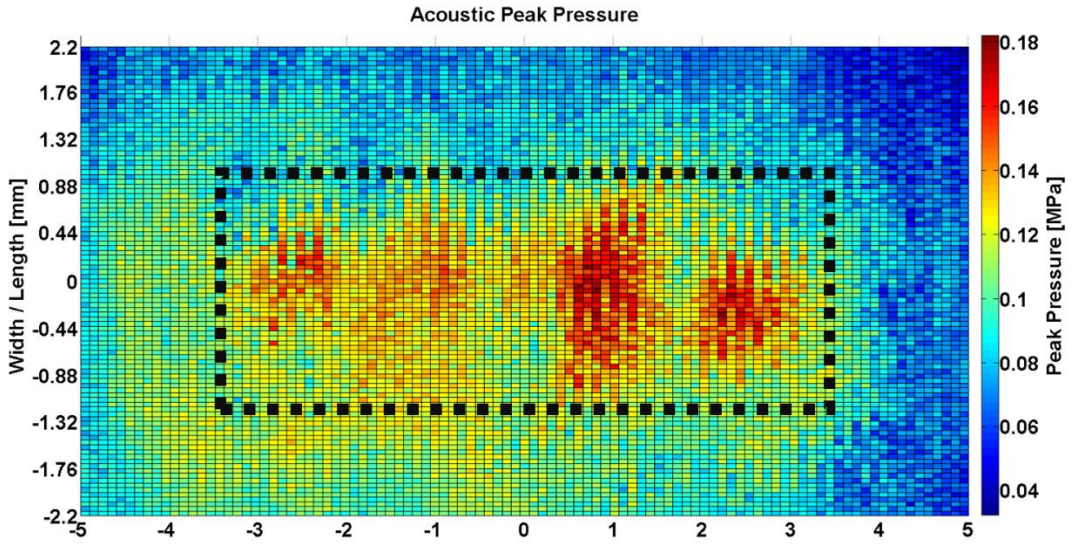


Figure 4.2 Measured acoustic pressure of 10 by 10 pMUT array


Acoustic Peak Pressure
Width / Length [mm]
Peak Pressure [MPa]
2.2
1.76
1.32
0.88
0.44
0
-0.44
-0.88
-1.32
-1.76
-2.2
-5
-4
-3
-2
-1
0
1
2
3
4
5
0.16
0.14
0.12
0.1
0.08
0.06
0.04


Acoustic Peak Pressure
Width / Length [mm]
Peak Pressure [MPa]
2.2
1.76
1.32
0.88
0.44
0
-0.44
-0.88
-1.32
-1.76
-2.2
-5
-4
-3
-2
-1
0
1
2
3
4
5
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04


Acoustic Peak Pressure
Width / Length [mm]
Peak Pressure [MPa]
2.2
1.76
1.32
0.88
0.44
0
-0.44
-0.88
-1.32
-1.76
-2.2
-5
-4
-3
-2
-1
0
1
2
3
4
5
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04

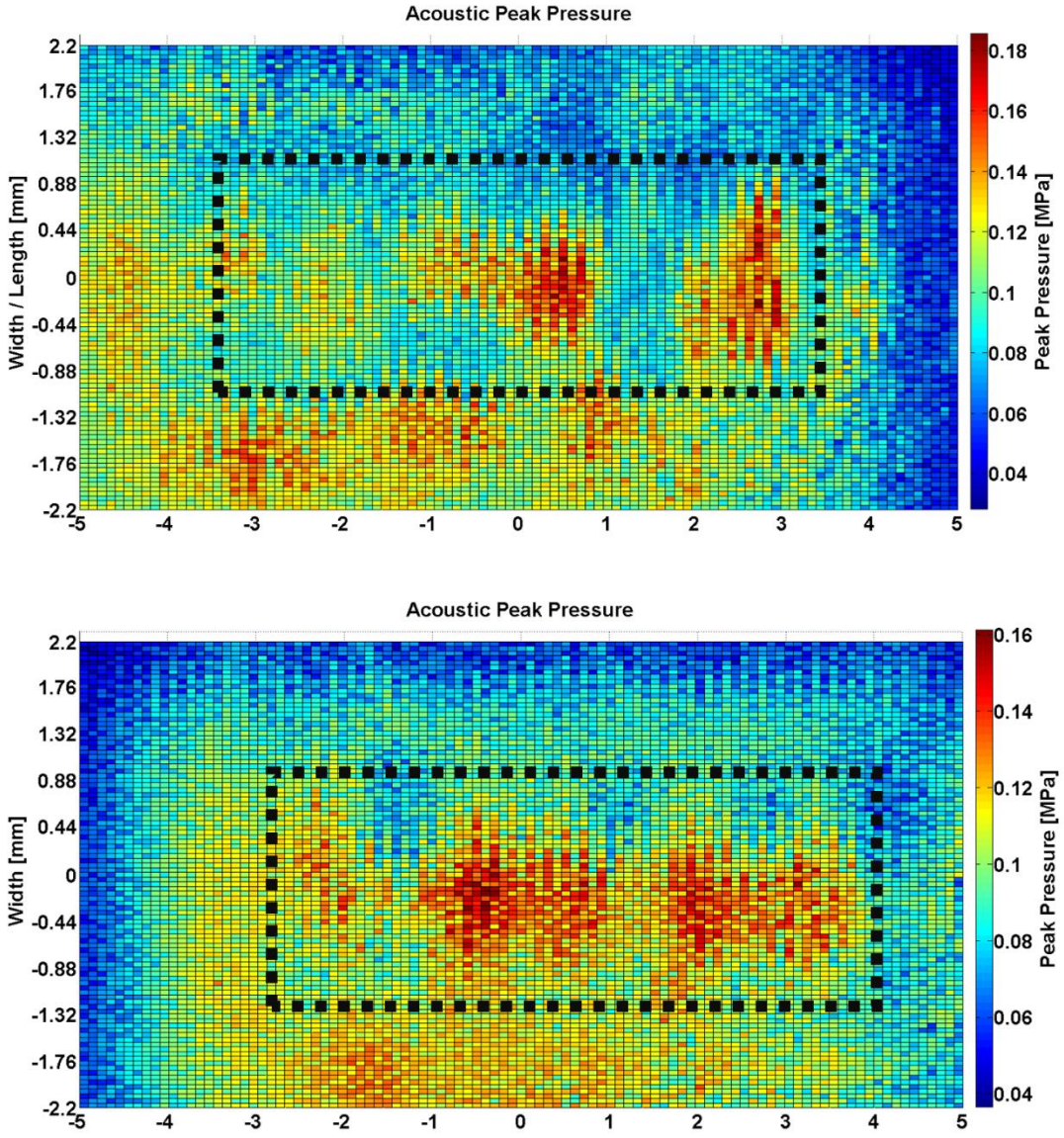


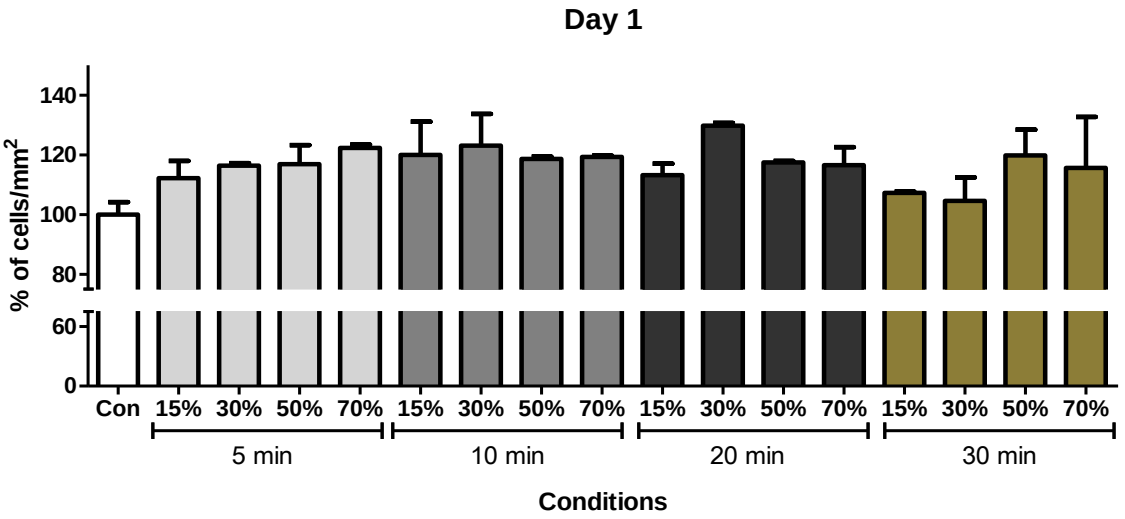
Figure 4.3 Measured acoustic pressure of 10 by 29 pMUT array

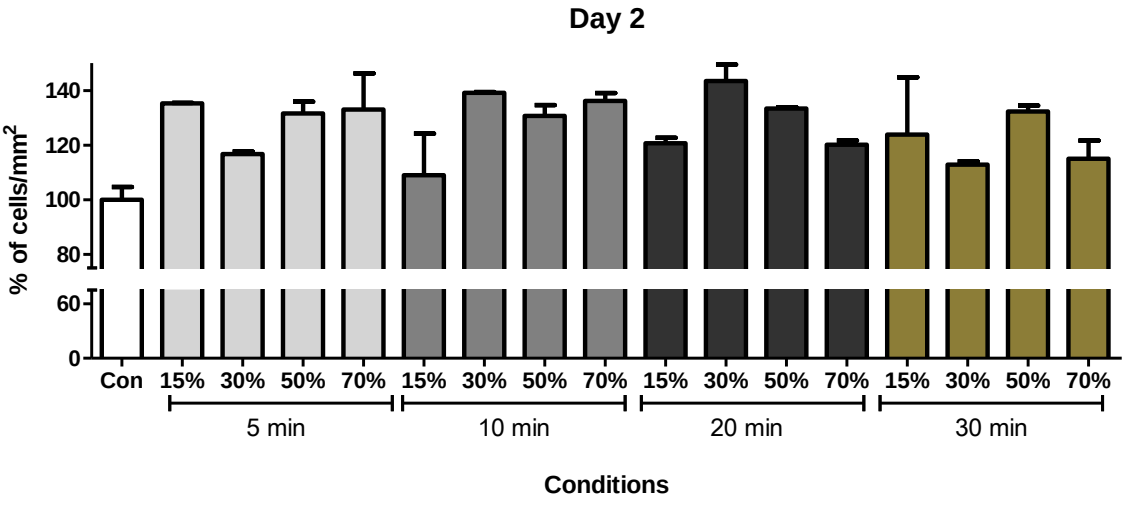
4.3 Results of the cell stimulation

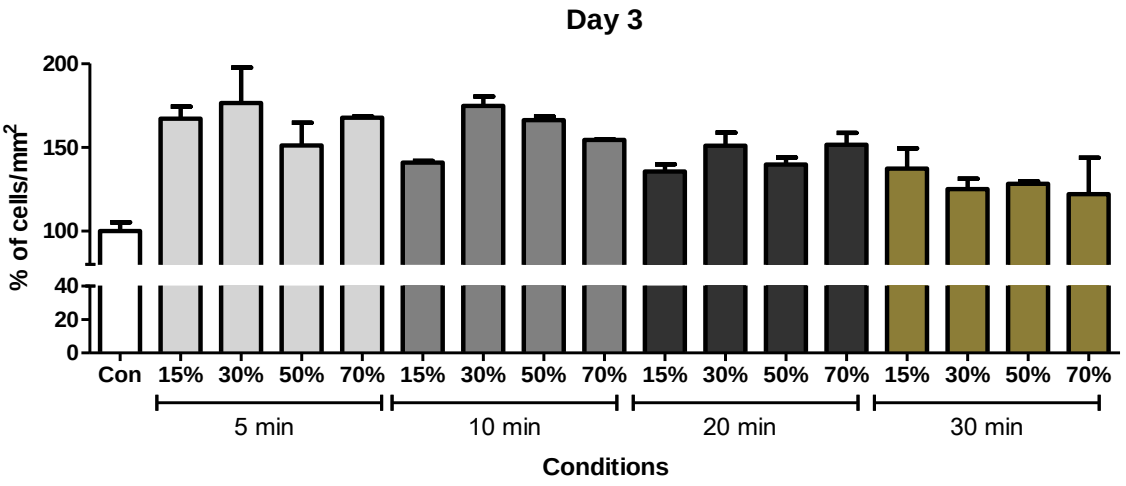
In previous studies, ultrasonic stimulation is thought deeply involved in the neuronal regulation and healing through many clinical trials. To characterize the neuro modulation effect of ultrasonic stimulation of underlying its core effect on brain activity enhancing, the result shown ultrasonic stimulation with modifying the duty cycle and time duration on neural progenitor cells (NPCs), PC12 cell, proliferation. To examine the enhanced proliferation effect by ultrasonic stimulation, an arranged different duty cycle (15 %, 30 %, 50 %, 70 %) and stimulation duration (5 min, 10 min, 20 min, 30 min) of ultrasonic stimulation were shown as the table 4.2.

Conditions	**Duty Cycle**			
	15 %	**30 %**	**50 %**	**70 %**
	No Stimulation			
Stimulation duration / Day	5 min	5 min	5 min	5 min
	10 min	10 min	10 min	10 min
	20 min	20 min	20 min	20 min
	30 min	30 min	30 min	30 min

Table 4.2 16 parameter was composed of combined to stimulation time and duty cycle for stimulation and 1 control group.


Day 1
% of cells/mm2
140
120
100
80
60
0
Con
15%
30%
50%
70%
5 min
10 min
20 min
30 min
Conditions


Day 2
% of cells/mm2
140
120
100
80
60
0
Con
15%
30%
50%
70%
5 min
10 min
20 min
30 min
Conditions



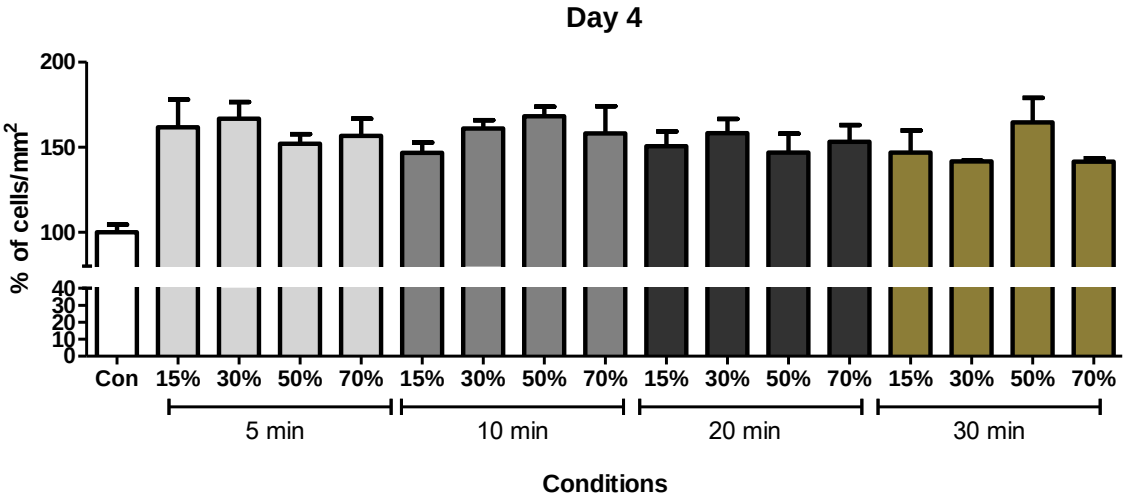


Figure 4.4 Each day incremental cell density percentage with 16 parameter compare the control group.

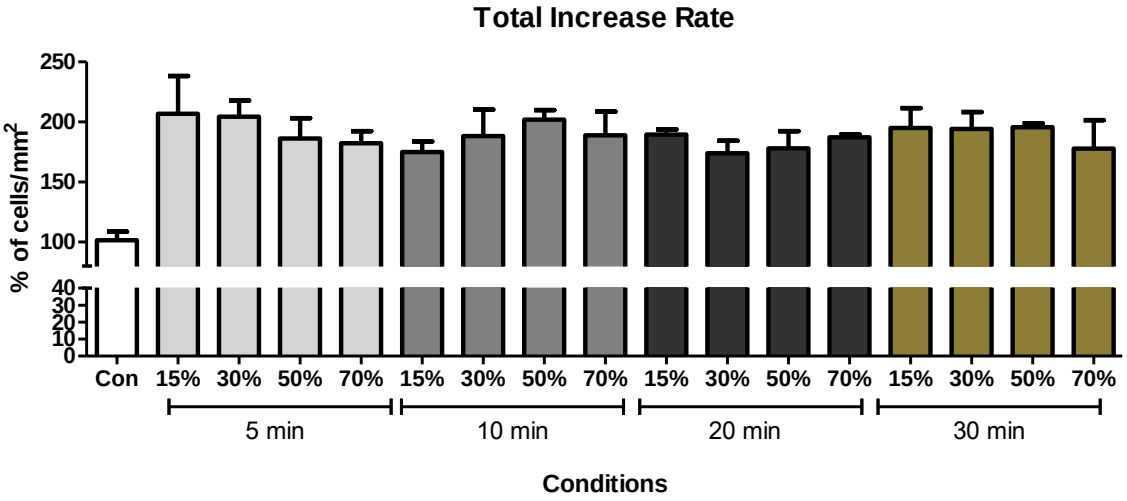
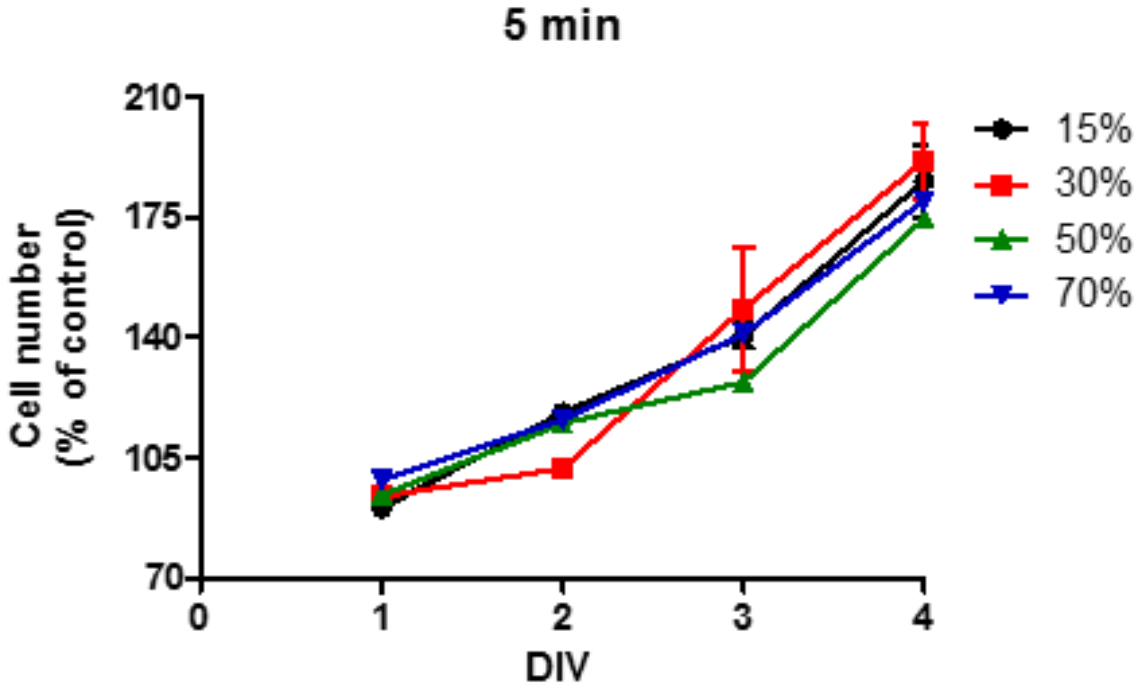
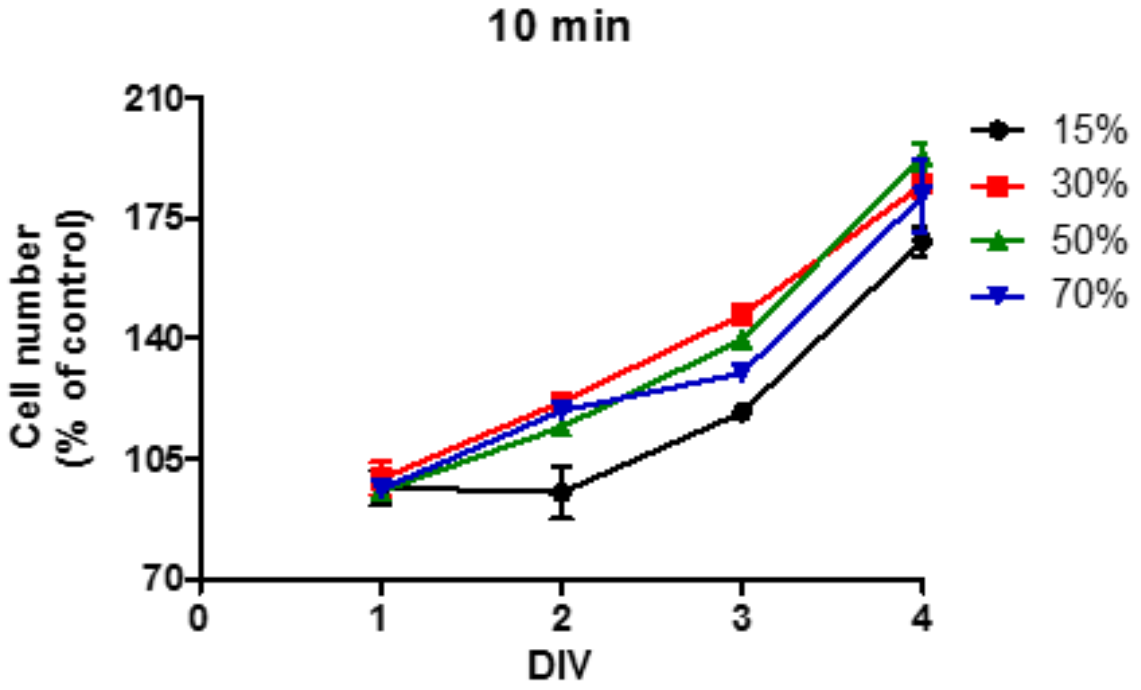


Figure 4.5 Total increase rate of cell density with 16 parameter and control group, especially 5 min 15 % group has most incremental in cell number.

In various conditions, ultrasonic stimulation induced addictive proliferation effect on PC12 cells as shown figure 4.4. Day 1 showed the counted cell number after seeding. Day 2 showed the counted cell number after first stimulation. After first stimulation, cell number increased average 17 %. Day 3 showed the counted cell number after second stimulation. After second stimulation, cell number increased average 13 %. Day 4 showed the counted cell number after third stimulation. After last stimulation, cell number increased average 43 %. Those figure presented irrespective of various conditions and there was increase in cell number than control group. The figure 4.5 was shown for totally increased cell number with 16 parameter and control group. In this figure, 5 min and 15 % parameter indicated 238 % increase rate and other group also shown 150 % average increase rate.


5 min
Cell number
(% of control)
210
175
140
105
70
0
1
2
3
4
DIV
15%
30%
50%
70%


10 min
Cell number
(% of control)
210
175
140
105
70
0
1
2
3
4
DIV
15%
30%
50%
70%

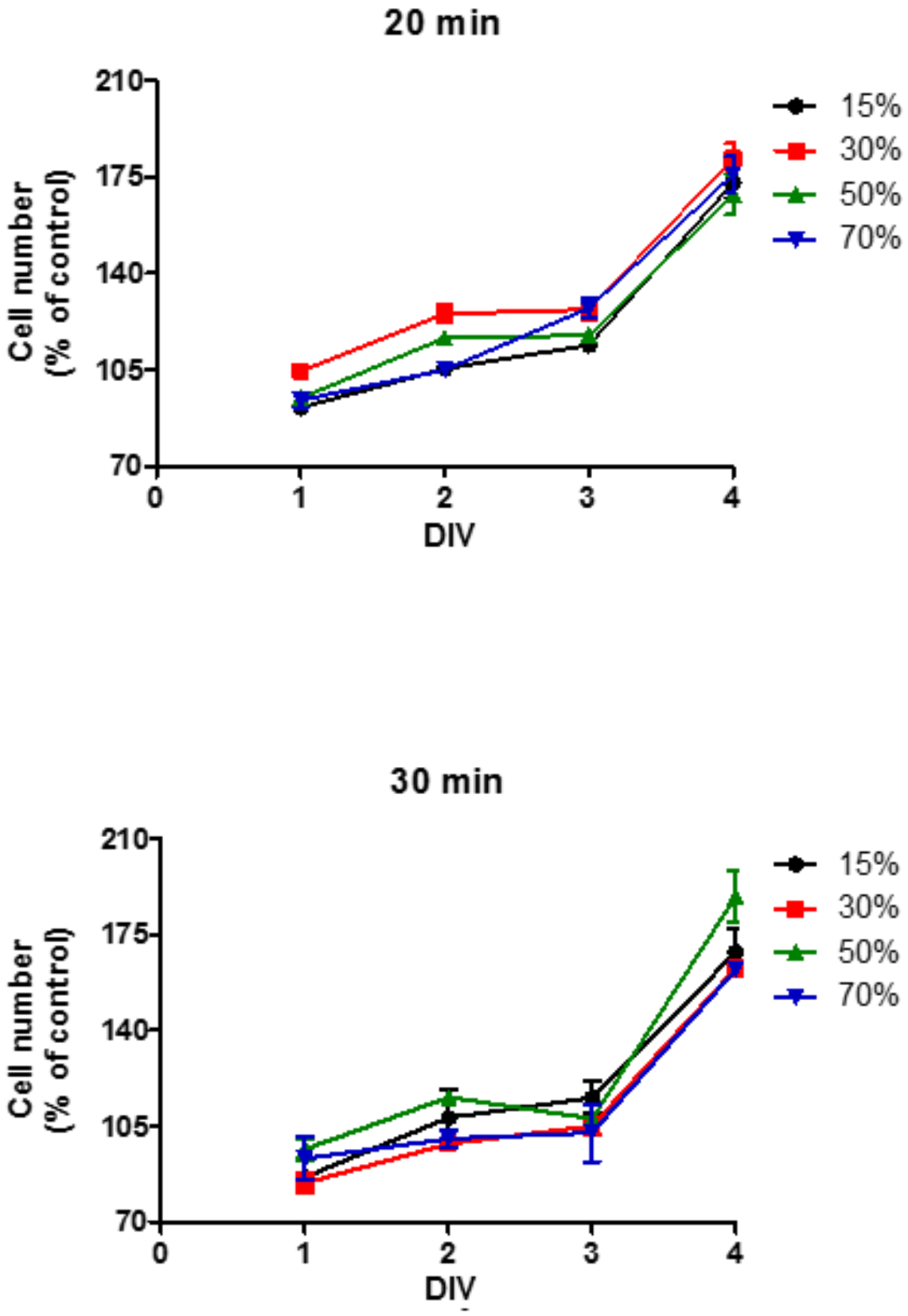
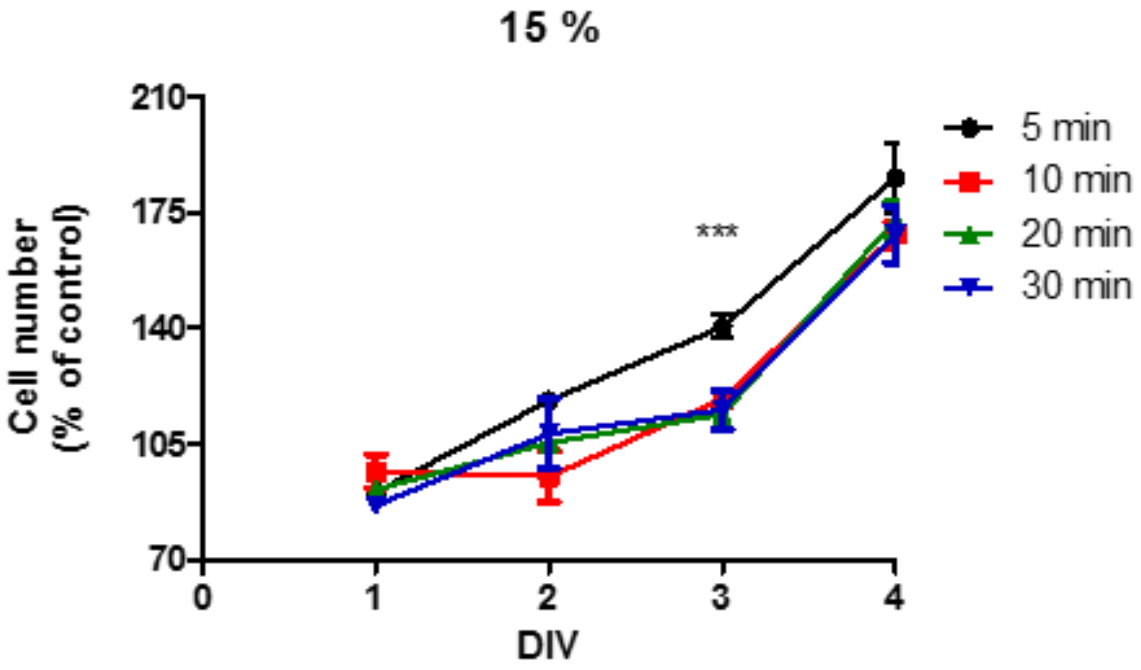
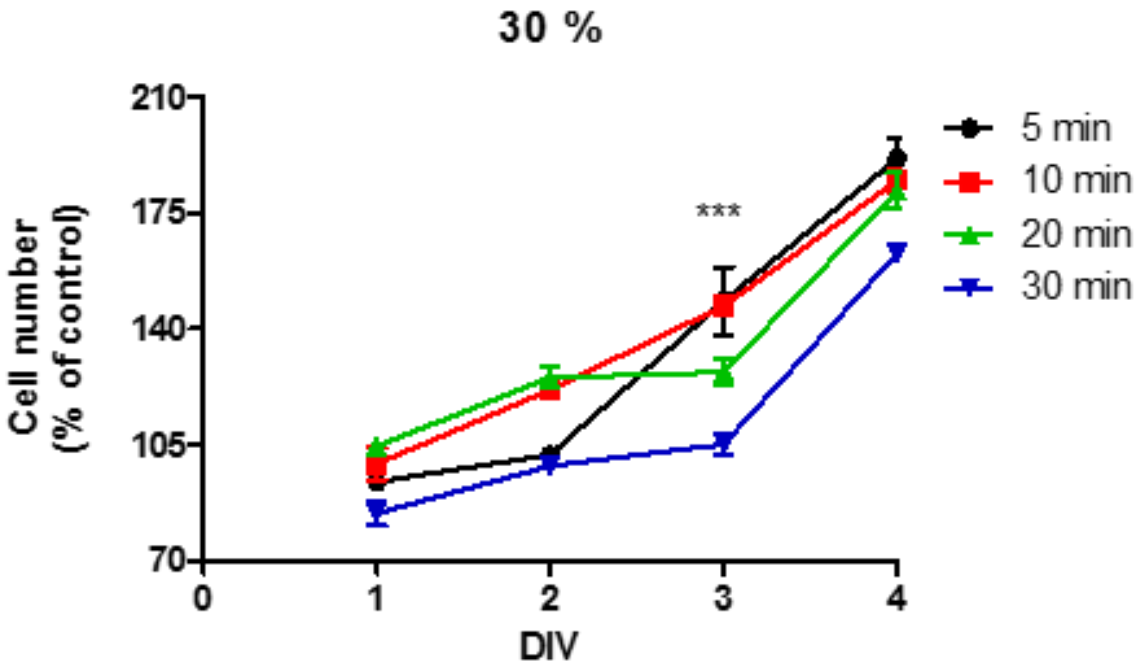


Figure 4.6 Results of the cell stimulation according to the several time duration, between 3 day and 4 day has dramatically increasing cell number in 20 min and 30 min.


15 %
210
175
140
105
70
Cell number
(% of control)
0
1
2
3
4
DIV
***
5 min
10 min
20 min
30 min


30 %
210
175
140
105
70
Cell number
(% of control)
0
1
2
3
4
DIV
***
5 min
10 min
20 min
30 min

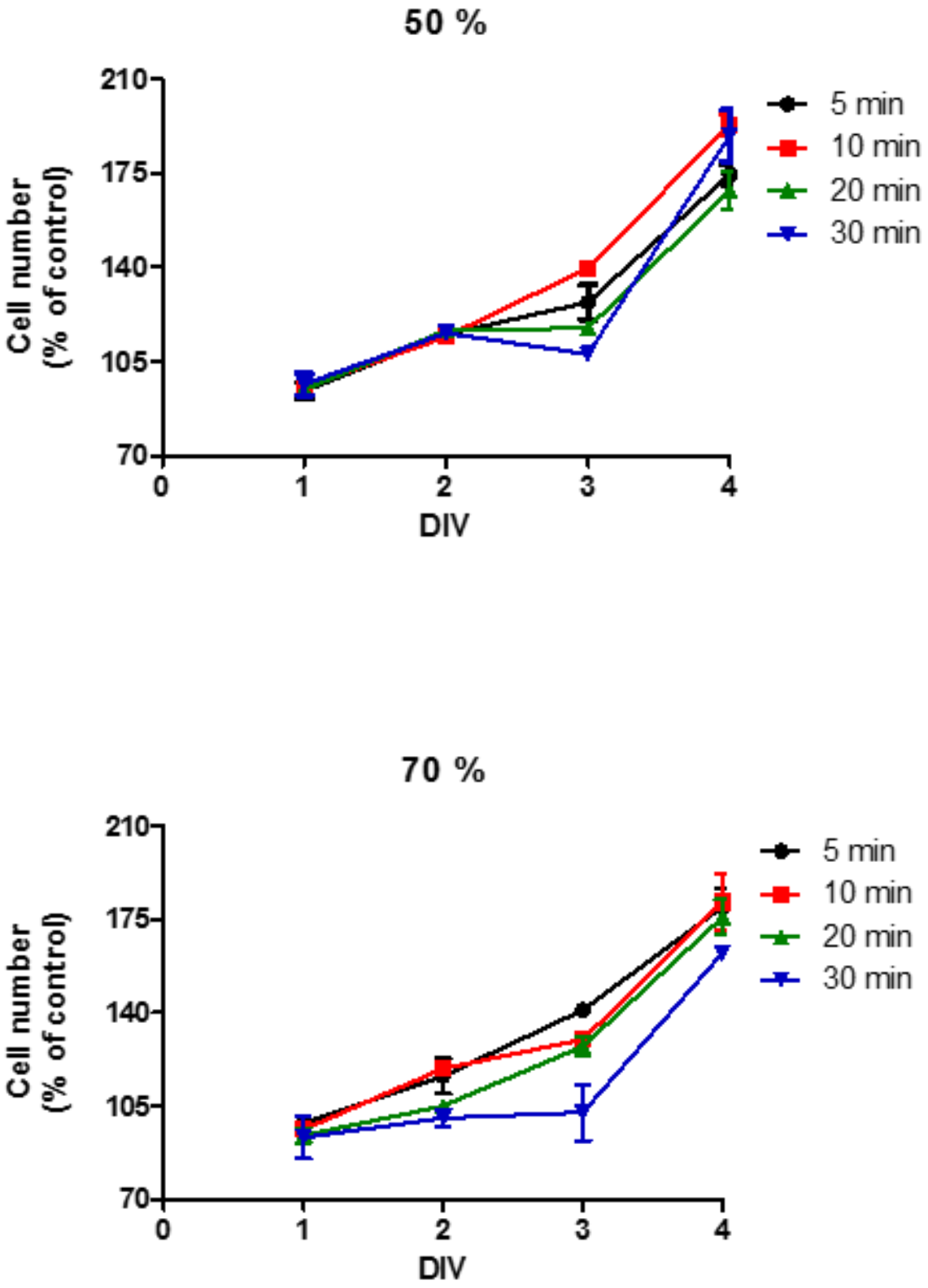


Figure 4.7 Results of the cell stimulation according to the several duty cycle, between 3 day and 4 day has dramatically increasing cell number in 20 min and 30 min.

Also, proliferation pattern was different with following time duration, not duty cycle. According to time duration, 5 min and 10 min had linear increment as figure 4.6. Those stimulation time group showed 118 %, 122 %, 134 % increase rate of cell number during each stimulation. However, in 20 min and 30 min had linear increment from 1 to 3 day as 117 % and 105 % but, these condition showed dramatic increment as 152 % from 3 to 4 day regardless of duty cycle. For more conformation, result of the cell stimulation was divided for duty cycle duration, it did not affect to proliferation for the cell as shown figure 4.7.

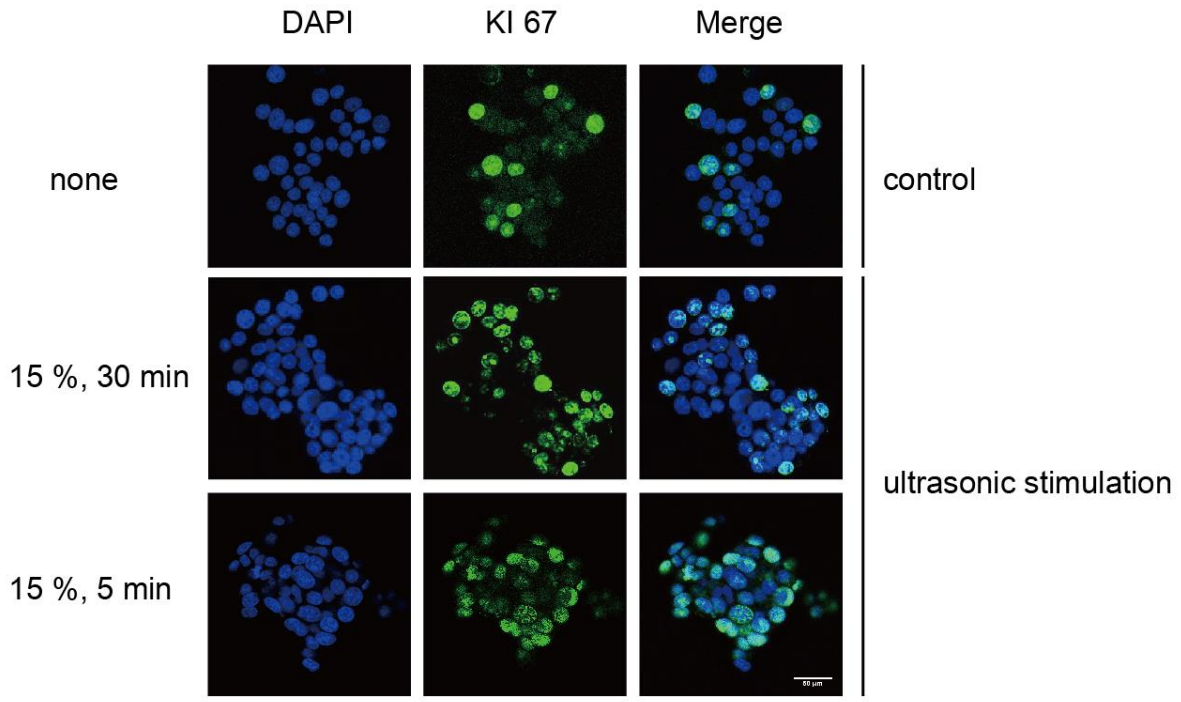


Figure 4.8 Fluorescence image of cell, compare ultrasound group to control group using Ki-67 and DAPI.

To evaluate the PC12 cells proliferation under different stimulation duration at specific duty cycle depends on day in days in vitro (DIV), it was measured 5 min duration and 30 min duration conditions. The results showed significant difference level of cell numbers in Day 3. To explore different level of cell number in day3, we adopt proliferation marker Ki-67 to distinguish the proliferative cells as shown figure 4.8. Compare to control condition, Ki-67 (+) cells increased in ultrasonic stimulation conditions. DAPI is used for nuclear dyeing and Ki-67 is used for indication of proliferative. Compare the ultrasound group and control group with similar number of cell, in the ultrasound group cells has more proliferative.

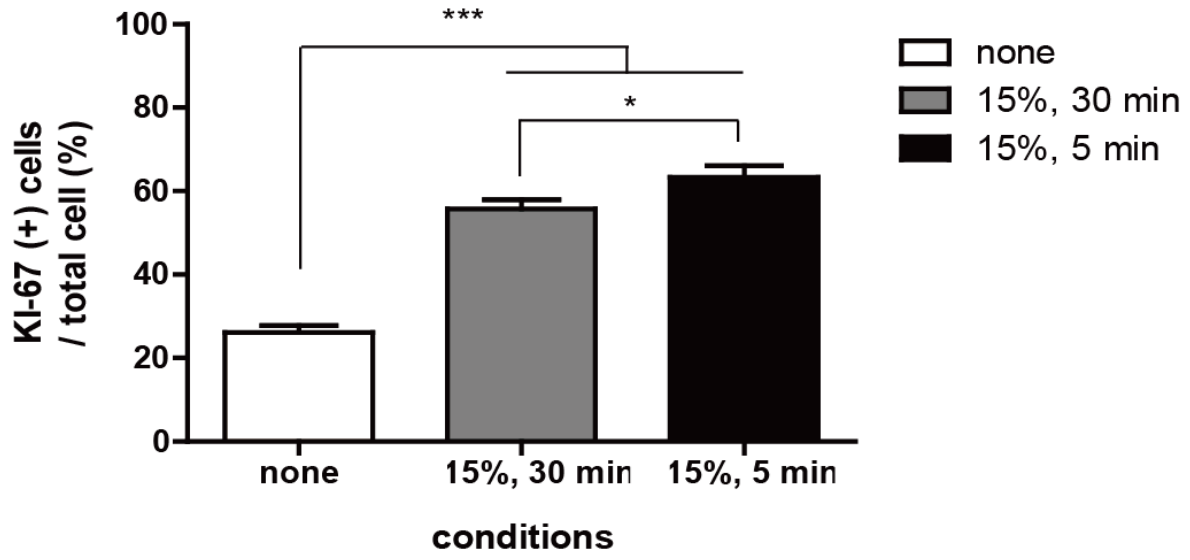


Figure 4.9 Compare the proliferative with control group and ultrasound group, the short time duration in more effective than long time duration.

Even within the ultrasonic stimulation conditions, number of Ki-67 (+) cells significantly high in 15%, 5 min condition compare to 15%, 30 min condition. Ki-67 positive means that the cell has the ability of proliferation. 15 %, 5 min group has 58 % of Ki-67 positive cells and 15 %, 5 min group has 62 % of Ki-67 positive cells. These data suggested that short time duration is more effective than long time duration (Figure 4.9).

4.4 Discussion

pMUT has several advantage than bulk transducer and cMUT, such as small size and applied low input voltage. Nevertheless, pMUT was used only for cell manipulation in bio research field. pMUT to use for medical area that need confirm to affect the positive effect to increase the rate of proliferation and differentiation. After, those fundamental research using the pMUT which will develop neural stimulation transducer and inserted transducer in human body. We made a 10 by 29 pMUT array and 10 by 10 pMUT array for cell stimulation that size was 2.27 mm by 6.84 mm and 2.5 mm by 2.5 mm.

The cell stimulation system used in this research that size was small than existing cell stimulation system. The system size was 13.4 X 17.3 X 2.5 cm that was enough size to enter the incubator. Also, we used 12 well-transwell to stimulation with several parameter at one time. Thus, we removed external environment and reduced the cell stimulation time. Therefore this research increased the reliability for the ultrasound cell stimulation experiment.

We measured the acoustic pressure of 10 by10 array and 10 by 29 array using the AIMS and obtained the 0.243 MPa and 0.161 MPa with 5 V_{pp} AC voltage applied. Those results are proper acoustic pressure for cell stimulation and this pressure are low intensity acoustic pressure[44]. In this research, we only applied 5 V_{pp} AC voltage, so the acoustic pressure was fixed. However we will obtaining the several acoustic pressure with several AC voltage. The acoustic pressure result figure was not clean because the ultrasound signal was reflected by water surface during the measure the acoustic pressure. Conventional measuring method for acoustic pressure, the transducer and hydrophone was located in water bass. This method reduced the reflecting acoustic signal from the water surface. However, in this

research, water was filled in completed system and hydrophone scan from on the pMUT array. For this reason, reflected ultrasound was occurred during the measuring acoustic pressure.

I just used 5 V_{pp} AC voltage for measuring acoustic pressure and cell stimulation. Thus the intensity was fixed from the pMUT array. The intensity of the pMUT was changed by AC voltage level. In the future research, acoustic pressure is measuring with several AC voltage level and obtain the several intensity of ultrasound. In the ultrasound cell stimulation research, acoustic intensity is most important parameter. The pMUT array need characterizing of several parameter to be use cell stimulation area.

After cell stimulation using PC12 cell, ultrasound promoted the proliferation of cell. I used 17 parameter with stimulation time and duty cycle. According to results, most effective parameter is 5 min and 15 %. Furthermore, the most important result that is ultrasound generates different mechanism to proliferation of cell according to simulation time. However, in this research I did not found mechanism why proliferation was promoted by the ultrasound. Future plan, I will make live cell observation system during the cell stimulating with ultrasound. This future research will solve the mechanism why ultrasound promote the proliferation of the cell.

5. CONCLUSIONS

Conventional cell stimulation system has limitation for the cell stimulation, it could not enter the incubator during the cell stimulation. This limitation reduce the reliability, because external environment was effected to the cell during cell stimulation. To overcome this advantage, in this thesis, the new cell stimulation system was developed using the 2D pMUT array and 12-well trnaswell. Ten wells were connected to each pMUT array and pMUT array stimulates the cell with different parameters respectively. Two well were used as control group. Fabricated 2D pMUT array has appropriate resonance frequency and acoustic intensity for the cell stimulation. Acoustic intensity of 10 by 10 pMUT array and 10 by 29 pMUT array were measured in water and intensities were 0.22 MPa and 0.18 MPa individually. Furthermore, it has 1.493 MHz resonance frequency.

In this thesis, PC12 cell are used for cell stimulation. Cell stimulation proceed for promoting the proliferation. During the cell stimulation, 17 parameter was applied. The simulation time and duty cycle has 4 parameters as 5 min, 10 min, 20 min, and 30 min, also 15 %, 30 %, 50 %, and 70 % of duty cycle. Those parameter was combined, as the result 17 parameter was made with one control group. After stimulation experiment, stimulated cell was promoted than control group. In this thesis, it obtained averagely 150 % increase ratio of cell number. All of the stimulation parameter was promoted for proliferation, especially, 5 min, 15 % parameter has most increase number of the cell as 238 %. However, it has still not been studied why proliferation of the cell is promoted by the ultrasound.

Proposed system used to investigate the effect of the ultrasound on cell stimulation with different stimulation parameter value. The new stimulation system has additional

advantages, which reduce the experimental cost and time consumption by stimulation simultaneously with 12-well transwell.

In the future, cell stimulation system using the pMUT will be designed for enhanced efficiency than this system and advanced system should changes the pMUT array shape and increase the number of well. Thus, it needs to find the parameter for cell stimulation using the ultrasound. In this thesis, the proliferation of the neuron cell was checked, for the future, the differentiation of the cell will be promoted when applied the ultrasound to the cell. Those results were fundamental research for treatment to neuronal disease. Furthermore, using the advantage of the pMUT, it can make a cell analysis system during the stimulation and this system will find the mechanism of the cell why the cell proliferates and differentiates by ultrasound stimulation.

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요 약 문

pMUT 과 transwell 을 이용한 세포의 활동성 증진을 위한 초음파 세포 자극 시스템의 개발

기존의 초음파를 이용한 세포 자극 시스템의 경우 벌크 세라믹 트랜스 듀서를 이용하여 만든 물리치료용 초음파 자극기를 이용하여 세포 실험을 진행 하였다. 이러한 자극기의 경우 가격이 비쌀 뿐만 아니라 크기가 커서 인큐베이터 안에서 작동을 하기에 어려움이 있다. 인큐베이터 안에서 세포 실험을 진행하지 못할 경우 외부환경에 세포가 노출되어 오염의 위험이나 외부환경의 유입에 의한 정확한 세포실험이 이루어 질 수 없다. 이러한 단점을 보완하고자 pMUT 을 이용한 세포 자극 시스템을 개발하였다.

pMUT 은 미세가공기술 (MEMS)을 이용하여 마이크로 단위의 트랜스 듀서이다. 특히 본 논문에서 제작된 pMUT 의 경우 Top cross over to bottom (TCTB) 라는 공정 기술을 이용하여 2D pMUT 어레이에서 원하는 부위의 트랜스 듀서만 개별적으로 작동 시킬 수 있게 만들어 주는 공정 기술이다. 또한 이 기술은 기존의 벌크 세라믹 트랜스 듀서가 가지고 있던 전기적 연결을 위한 와이어링 문제도 해결 하였다. 본 연구에서는 120μm 의 직경을 가지는 트랜스 듀서를 제작하였으며 1.5 MHz 의 공진 주파수를 갖게 제작하였다. 제작된 pMUT 의 공진 주파수는 기존연구에서 세포자극으로 쓰이는 주파수 영역이다. 가로세로 각각 10 개, 29 개, 10 개, 10 개로 제작된 pMUT 어레이를 이용하였으며 세포 배양과 자극을 위해 12 개의 배양 접시를 가지는 트랜스웰을 이용하였다. 이 트랜스웰의 경우 세포배양을 위한 내부 웰이 따로 존재하여 세포의 배양을 좀 더 쉽게 해 주었다. 트랜스웰의 크기에 맞게 PCB 를 제작하여 12 개의 배양접시의 중앙에 각각 pMUT array 가 위치하게 제작하였다. 트랜스웰의 바닥을 모두 제거하여 기존의 세포 자극 시스템의 사용시 초음파 강도의 감쇄 문제를 해결 하였다. 또한 pMUT 이 부착된 PCB 를 방수와 생체적합성을 위해 페릴렌 C 로 코팅을 하였다. PCB 와 바닥이 제거된 트랜스웰은 에폭시와 PDMS 를 이용하여 접착하였다.

제작된 초음파 세포 자극 시스템의 성능을 평가하기 위해 AIMS 를 이용하여 pMUT 어레이에서 발생되는 음압과 강도를 측정하였다. 측정결과 10 by 10 pMUT 은 0.25 MPa ± 0.002 MPa 10 by 29 pMUT 은 0.18 MPa ± 0.03 MPa 음압이 측정되었다. 특히 10 by 29 pMUT 어레이의 음압은 강도로 변환 하였을 때 200 ± 22 mW/cm^2 의 강도로 변환 되었다. 이러한 강도와 음압은 기존의 세포 실험에서도 사용 되었다.

제작과 평가가 끝난 초음파 세포자극 시스템을 이용하여 PC12 세포의 분열을 촉진 시키는 연구를 수행하였다. 세포 실험에는 4 개의 자극 시간과 4 개의 자극 주기의 조합으로 비교군 포함하여 17 개의 파라미터로 실험을 진행하였다. 전체 실험은 총 2 번씩 진행 되었다. 세포자극 결과 초음파 자극을 받은 모든 그룹의 분열이 촉진되어 컨트롤 그룹보다 대략 150 %의 증가율을 보였다. 그 중에서도 5 분 15%의 자극 조건이 238 %의 증가율로 가장 좋은 반응을 보였다. 또한 크게 자극 시간과 자극 주기의 파라미터를 주었는데 그 중에서도 자극 시간에 따라 분열의 메커니즘이 다른 것을 확인하였다. 5 분과 10 분처럼 짧은 시간의 자극과 20 분, 30 분처럼 긴 시간의 자극에서의 결과가 달랐다. 짧은 시간의 경우 날짜의 증가에 따라 118 %, 122 %, 134 %로 선형적으로 세포가 증가하였으나 긴 자극에서는 1 일에서 3 일 까지는 평균 12 %의 증가율을 보였으나 3 일에서 4 일째 자극에서 세포의 수가 급격히 151 % 증가하는 것을 확인할 수 있었다. 이러한 결과를 바탕으로 시간에 따라 세포의 분열에 영향을 미치는 메커니즘이 다르다는 것을 확인 할 수 있었다. 추후 연구를 통해 본 논문에서 소개된 시스템과 pMUT 을 이용한다면 정확한 메커니즘 규명과 초음파를 이용한 세포 자극 파라미터를 확립 할 수 있을 것으로 기대가 된다.

핵심어: pMUT, 세포 자극 시스템, 12 웰 트랜스웰, PC12 세포, 세포분열

APPENDIX

Acoustic intensity measurement method using the AIMS

(1) Clean the inside of water tank

(2) To fill up the water tank with DI water

(3) Turn on the computer and stage controller

(4) Turn on the SONIQ program

(5) Select the hydrophone type (HNC-1000) in the program

(6) Fix the hydrophone with clamper that is attached water tank

(7) Put the size of transducer and resonance frequency in the program

(8) Calibrate the location of hydrophone

(9) Fix the transducer with clamper that is attached water tank

(10) Move the hydrophone toward desired position (center of transducer)

(11) Reset the now hydrophone position in the program. If you do not do that, hydrophone and transducer may crush.

(12) Turn on the transducer and check the signal wave in oscilloscope

(13) Select the intensity type in the program

(14) Select the 1D, 2D and 3D scan type in the program

(15) Determine the scan area(X, Y and Z axis).

(16) Determine the scan point(X, Y and Z axis). Ex) in 2D scan 100 by 100 point is reasonable.

(17) Click the ‘start’ button.

Cell stimulation using pMUT

(1) To sterilize cell stimulation system use the 99.9 % alcohol during overnight

(2) Inserted well coated by 0.01% poly-L-lysine (Sigma-Aldrich, USA) during overnight

(3) Media used Roswell Park Memorial Institute medium supplemented with 10% horse serum, 5% fetal bovine serum (Hyclone, Thermo Fisher Scientific, USA) and 1% antibiotics, at 37˚C in 5% CO_2.

(4) Seeding the PC12 cells (ATCC, USA) on inserted well for 1.0×10^5 cells/ml

(5) Setting the incubator environment at 37˚C in 5% CO_2

(6) After seeding, stabilize for 1 day

(7) Input signal was applied by function generator (33500B, Agilent, USA)

(8) Setting the input voltage as 5 V_{pp} square wave, duty cycle and resonance frequency

(9) After sterilization of cell stimulation system, clean the system using the sterilized water four times.

(10) After washing, dry and sterilize using the UV

(11) Fill a half of well with media (10% horse serum, 5% fetal bovine serum (Hyclone, Thermo Fisher Scientific, USA) and 1% antibiotics)

(12) Inserted well, which included cells, is transferred to cell stimulation system from the 12-well

(13) Put in the cell stimulation system to incubator

(14) Turn on the function generator and timer

(15) After cell stimulation, remove the media using suction in the clean bench

(16) After remove media, cell stimulation was sterilized by alcohol during overnight