University of Alberta

Exploring Novel Methods in Sonoporation

by

Peter Kwok Pan Wong

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Biomedical Engineering

Fall 2012
Edmonton, Alberta

Abstract

Sonoporation, a physical, non-viral, non-chemical transfection method, promises great potentials. However, many drawbacks hinders its generalization. Low transfection rate and cell viability after treatment are among the hindering factors of sonoporation.

The purpose of the research performed in this thesis was to develop, explore and analyze new methodologies to overcome the known drawbacks of sonoporation, which are to increase cell viability and transfection rate. These novel methods include the use of a self-developed ultrasound box, self-developed microbubble carriers and the synergistic use of chemical transfection reagents. Sonoporation were performed on MCF-7 and KG-1 cells as they represent easy and difficult to transfect cell lines respectively. Permeability markers, flow cytometry, MTT assay and MTS assay were used to quantify transfection rate and cell viability after sonoporation.

New procedures were performed, analyzed and evaluated for their feasibility for drug and or gene delivery. The thesis has shown improvements in transfection rate and preserving viability. However, sonoporation still remains an inefficient method to deliver material into hard-to-transfect cells.

Acknowledgement

I would like to give my gratitude to many individuals who have encouraged, guided and assisted me throughout my research project.

I give my uttermost gratefulness to my supervisor, Dr. Jie Chen. He has giving me valuable experience to work with him and participate in this interesting thesis project. Dr. Chen has given me encouragements and directions when I was in despair and lost. I would also like to thank Dr. James Xing, Dr. Hilal Gul-Uludağ, Dr. Lawrence Le and Dr. Quanrong Gu for their valuable advice and insightful comments. My thanks also extend to Dr. Alan Wilman and Ms. Maisie Goh for their administration assistance and support.

I would like to acknowledge my fellow lab members, Ms. Ming Huang, Mr. Ming Li, Mr. Iwain Lam, Mr. Chuan He for their warmest support. Special thanks to Ms. Xiao Yan Yang and Mr. Peng Xu for their patience in teaching and guidance. Mr. Woon Ang and Mr. Michael Choi have lent me their technical knowledge and skills in troubleshooting the ultrasound machines.

During this work, I have collaborated with many colleagues to whom I extend my warmest thanks: Ms. Dorothy Kratochiwil-Otto, technical manager at the Flow Cytometry Facility at the University of Alberta and Dr. Xuejun Sun, supervisor at Cell Imaging Facility at Cross Cancer Institute.

Finally, I appreciate the financial support from Queen Elizabeth II Graduate Scholarship for funding part of my research.

Table of Contents

Chapter 4 - Results and Discussion

List of Tables

List of Figures

List of Symbols, Nomenclature, or Abbreviations

CT	Computer Tomography
MRI	Magnetic Resonance Imaging
PET	Positron Emission Tomography
RNA	Ribonucleic Acid
kbps	kilo base pairs
PEI	Polyethlyenimine
DNA	Deoxyribonucleic Acid
DC	Duty Cycle
FDA	Food and Drug Administration
PESDA	Perfluorocarbon- exposed sonicated dextrose albumin
HPSC	Hematopoietic Stem Cells
ATCC	American Type Culture Collection
IMDM	Iscove's Modified Dulbecco's Medium
BGS	Bovine Growth Serum
rpm	rotations per minute
DMEM	Dulbecco's Moified Eagle Medium
FBS	Fetal Bovine Serum
siRNA	Small interfering RNA
GFP	Green Fluorescent Protein
FITC	Fluorescein Isothiocyanate
P/S	Penicillin and Streptomycin
hCMV	Human cytomegalovirus
HTLV	Human T-lymphotropic virus
UTR	Untranslated Region
SV40 pAn	Simian Virus 40 polyadenylation signal
LB	Luria-Bertani
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
BSA	Bovine Serum Albumin
DAPI	4',6-diamidino-2-phenylindole
SSC	Side Scattering

FSC	Forward Scattering
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
OD	Optical Density
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope

Chapter 1
Background Information and Literature Study

1.1 - Gene Therapy and Drug Delivery

As our understanding of cellular biochemistry increases, coupled with advances in pharmaceutical technologies, many of the well-established but non-specific drugs are being phased out gradually. Research is now focused on developing specific drugs that target unique indicators of desired cells [1]. These new and highly specific therapies take advantage of the subtle differences internally and externally between cells. Differences between cells begin at the surface. Every type of cell has unique membrane proteins, which are their identifiers. Drugs can be placed inside carrier molecules which target these specific surface proteins and receptors, and in turn, allow them to be selectively delivered to the desired location in the body. Furthermore, cells have specific enzymes, which are affected by certain pharmaceuticals. Enzymes and proteins targeted by the drug are usually part of an upstream pathway such that the drug alters the cell's physiology; other cells without the specific enzymes are unaffected and unharmed. These mechanisms provide selectivity to which cells can be targeted.

Researchers have long known that anti-cancer drugs destroy both cancerous and non-cancerous cells. An ideal smart drug would attack only cancerous cells, leaving non-cancerous cells unharmed. The challenge in oncology research is to develop drugs that can differentiate between two very similar cells. The difference between a cancerous and a non-cancerous cell can be as little as the difference in the DNA sequence of one gene. Diagnostic technologies can help identify cancerous cells through a different approach.

The most common technologies for locating cancerous cells include X-ray/CT, MRI and PET scans [2]. X-ray based technologies, such as computed tomography (CT), detect

morphological changes of tissue. As x-rays pass through tissue, it is attenuated by absorption and scattering processes. Different tissues have different densities, causing different attenuation properties. Identification of morphological changes in tissue can signify potential cancerous areas. MRI (Magnetic Resonance Imaging) and PET (Positron Emission Tomography) scan detect functional changes in cellular activity. Cancerous cells replicate much faster than somatic cell. Tracking the oxygen or radioactive glucose usage by MRI and PET scan respectively can suggest potential tumor sites. These morphological and functional irregularities are being assessed for targeted delivery of cancer drugs. Carrier molecules or special coatings which are easily absorbed by tumor cells have been examined for their efficiency in site-specific delivery of drugs or radiotherapy enhancement reagents [3].

Gene therapy represents a powerful alternative to non-specific drugs for cancer therapy. By implanting a set of functional genes into cancerous cells, proper cellular function can be restored. Restoration of these genes' functions can effectively halt cancer progression [4].

In order for gene therapy to present real hope to cancer patients, gene delivery must be safe, efficient, and robust. Safe and effective transfection is required for both academic research and clinical trials. In academia, research on gene functionality, proteins, and signaling pathways rely heavily on the possibility of delivering genes in-vitro to targeted cells. Success in the laboratory can be transferred to clinical trials where cells with manipulated genomes will be placed inside organisms. Safe gene delivery with high cell viability and no long-term side effects is the foundations for many biological fields and also the goal of this thesis.

1.2 - Transfection Methodologies

Transfection is the process whereby foreign genes are inserted into a recipient eukaryotic cell [5]. Numerous techniques have been developed to insert desired genes into targeted cells. There are three general categories of transfection methodologies: viral, chemical, and physical. Each methodology has unique advantages and disadvantages.

1.2.1 - Viral

A virus is a small organism that does not contain a nucleus or any gene replicating mechanisms. A virus contains shell proteins, surface receptors, genome, and sometimes RNA (ribonucleic acid) polymerase. To multiply, a virus first has to infect a host and hijack the replicating machinery to produce their own essential shell and genome.

The virus attaches itself to the membrane of the target cell by either puncturing the cell membrane and inserting its genome into the cell, or entering the cell by endocytosis and releasing its genome inside the host. Once the genome is present in the cell, the host's ribosome will indifferently translate the foreign RNA into proteins. These proteins are used to create more virus particles inside the host cell.

Scientists have found ways to exploit the gene deposition ability of a virus. By modifying a well-known existing virus, the viral vector can be used to deliver genes to host cells. Viral genes have two phases after hijacking the host: transient and stable transfection. Transient transfection is when the host's physiological mechanisms are forced to help reproduce the virus inside until the host is destroyed. It is possible that the viral genes can be inserted into the host's genome without being activated, hence preserving the cell. This silence of viral activity, which allows cells to proliferate and pass on the transfected gene to the next generation, is called

stable transfection and is required for successful gene therapy. In successful gene therapy, the infected cells are not harmed, but cured through translation of the desired genes only.

Viral vector is extremely effective in delivering genes into cells. It is widely used because of its effectiveness in multiple cell types. Although the transfection rate is the highest among all gene delivery methodologies, there are drawbacks.

There are some unavoidable limitations when using viral transfection. When using viral transfection, the size of the targeted gene is limited to four to seven kilo base pairs (kbps) due to viral vectors ranging from 450 nm to 20 μm [6]. A viral particle may be large enough to contain a single gene, but cannot carry multiple gene sequences which tend to be larger than seven kbps. Viral particles tend to bind non-specifically to mammalian cells, therefore when viral transfection vectors were injected in vivo, transfection can occur throughout the body. This could lead to DNA transfection in non-targeted cells, which may cause unwanted and unforeseen physiological effects in viral receptive cells.

These limitations can be overlooked in ex-vivo studies. However, one further challenge remains: using a virus as a transfection vector cause the insertion of unwanted viral genes into a host cell. Even though the virus vector was modified to knock-out its infectious ability, the virus vector retains many of its original genes and is inserted into the target cells along with the desired gene. Should an unwanted viral gene insertion occur in the target cells' genome, the extra viral gene could recombine with the previously transfected viral genes, thus becoming active. This can cause the target cells to become an active viral host, resulting in their destruction as well as the spread of the viral particles throughout the body. This has obvious implications in clinical trials where patient safety is paramount. In past clinical trials, some patients who were implanted with viral transfected stem cells died [7]. As a result, this delivery method is no longer used in clinical trials.

Therefore, other transfection methodologies tend to be favoured.

1.2.2 - Lipoplexes and Polyplexes

Chemical complexes such as Lipoplexes and polyplexes are DNA and cationic lipid or DNA cationic polymer complexes, respectively, facilitate transfection [8]. There are many types of commercially available chemicals capable of forming lipoplexes with DNA. These chemical complexes present a safe alternative to viral transfection as they insert only the desired gene into the cells. These chemical transfection agents exploit the properties of the cell membrane as their delivery mechanism.

The cell membrane is composed of phospholipid bilayer, containing two layers of amphiphilic molecules. The phospholipids in the bilayer have a phosphate head and a lipid tail. The head is a charged hydrophilic molecule linked to a hydrophobic tail of carbon chains. Due to the hydrophobic and hydrophilic properties of these amphiphilic molecules, they form two layers of molecule where the phosphate head will face outwards and the carbon chains face each other. The bilayer has a hydrophilic shell and hydrophobic interior. The cell membrane acts to keep out unwanted molecules and ions. With facilitated transport, cells regulate the flow of material in and out of the cell.

DNA possesses hydrophobic and hydrophilic characteristics, and therefore, cannot easily pass through the cell's lipid bilayer. Significant amounts of DNA could not be transfected into a cell without the use of lipoplexes or polyplexes. Some of these amphiphilic chemicals can form a capsule around the DNA, encapsulating the gene. The cationic and hydrophilic nature of the lipoplex and polyplex capsule enables the complex to be inserted into a cell. As the shell of the positively charged carrier attaches, fuse and align itself with the negatively charged cell membrane, the capsule opens up on the inside of the cell, releasing the DNA into the cytoplasm.

Other amphiphilic chemicals may just bind with DNA to form DNA complexes. Once DNA is bound to a lipoplex or polyplex, the complex can be embedded into the membrane and is then taken in to the cells via endocytosis. One example would be polyethlyenimine (PEI), which increases the rate of transfection by forming complexes with DNA and enable the complex to attach to the cell membrane, waiting for endocytosis [9]. Neither pathway is mutually exclusive of each other and can occur together [10].

Even though it appears that chemical transfection agents cause no long term physical or physiological changes to cells, the cells do experience immediate adverse effects when exposed to chemical transfection agents. Transfection efficiency using these cationic chemicals is very dependent on the cell line. Some cell lines experience efficiencies of over 90% while some cell lines have efficiency of less than 3%. The mechanism leading to the variance in efficiencies between cell lines is mostly unknown. Such variance in transfection susceptibility and toxicity is the greatest drawback for the chemical transfection. Moreover, specific localization of transfection is not possible. As with viral transfection, these DNA-binded reagents enter the body, and deliver the genes to all parts of the body in a non specific manner.

This method of transfection has many advantages over viral transfection, including patient safety. The inserted DNA contains only desirable genes, which means that insertion of non-desirable viral genes is not a concern. Although lipoplexes and polyplexes are becoming the benchmark for transfection methodologies, they are, by no means, perfect. Efforts have been placed to reduce their toxicity and understanding how the complexes' structures influence uptake [11].

1.2.3 - Nanoparticles

In the past decade, use of nanotechnologies has advanced greatly, and the possibility of using nanotechnologies has been explored rigorously. Improved specificity for transfection in cellular biology is where nanotechnologies have had the greatest impact. By definition, nanoparticles are materials that have the size smaller than 100nm [12]. Nanoparticles can be employed using both chemical and physical means for gene transfection or drug delivery.

These nanoparticles are composed of biocompatible material such as carbon, silicon, and gold as a shell wrapping around a core. The core can be ferromagnetic metals or other materials which might be toxic or harmful to cells [13]. These biocompatible materials are used to coat the nanoparticles to protect the cells against harmful effects of the core material. These biocompatible nanoparticles are used as DNA carriers. These carriers have properties of being soluble, easily taken in by cells and are able to protect DNA from degradation. Nanoparticles can be coated with molecules, ligands and compounds to enhance their solubility and/or cellular uptake.

Specific antigens, antibodies or compounds can be attached to the surface of a nanoparticle, allowing them to selectively target cells [14]. Cells that have receptors for these antigens, antibody or compounds will bind these nanoparticles on their surface. After the nanoparticles are attached to the surface of the cell, it then follows the mechanism of other chemical-DNA complexes: endocytosis.

The material of the core itself can help improve transfection. Nanoparticles with a ferromagnetic core will respond when exposed to magnetic field. Strategically placing magnets behind targeted cells, it will cause the magnetic-nanoparticles to move in the direction of the magnetic field and onto the cells. This facilitates the attachment of nanoparticles to the cells surface and allows more nanoparticles to be taken up through endocytosis. Furthermore, it is believed to be possible that these magnetic nanoparticles can penetrate through the cell

membrane and arrive in the cytoplasm without going through endocytosis. This direct penetration increases transfection efficiency. After entering the cell, these nanoparticles are too small for cells' defence mechanisms to clear out of their cytoplasm effectively, thereby increasing the chances of transfection or delivery.

Nanoparticles hold much promise as a transfection vector and a drug delivery methodology. It has lower cytotoxicity than other transfection reagents and can selectively target cells. However, the cost and low transfection efficiency compared to viral transfection continues to hinder the commercialization of the methodology.

1.2.4 - Gene Gun

The gene gun is a non-viral, non-chemical transfection technique that uses physical force to induce transfection in cells. As the name implies, the gene gun shoots a gene into the target cells. DNA itself cannot be directly shot into the cells due to the extreme forces involved; rather they are bound to heavy metal pellets such as silver, gold or tungsten [15]. The heavy metal pellet and DNA complex are then shot into recipient cells using compressed gas.

One advantage of this physical transfection methodology is that most cell types can be successfully transfected. Furthermore, with the gene gun, localized transfection is achievable, allowing greater spatial specificity within cell culture. Excess pellets from the transfection process that are not injected into a cell will not transfect other cells. Specificity is achieved because of the large force required to deliver the DNA-metal complex into the cell. The localization of transfection by the gene gun is a well established technique and has been used to label cells within the same culturing dish.

The low viability after treatment and the lack of penetration power of transfection are the limitation of a gene gun. The DNA- metal complexes are unable to penetrate deeply into

tissue, therefore it can only transfect tissue on the surface such as skin [16]. New developments of the gene gun have shown to be able to transfect liver cells in murine species [17].

1.2.5 - Electroporation

Electroporation is another non-viral, non-chemical transfection methodology that has been used for decades. This method uses repetition of electric pulse shocks to induce a reversible breakdown of the cell membrane, increasing the permeability for exogenous molecules [18]. Electroporation can deliver drugs, genetic material, as well as other foreign material into a cell.

As with other physical methodologies, electroporation is effective for many different types of cell lines, from plant to bacteria to mammalian cells. The formation of the pore is thought to be due to the charged properties of the cell membrane [19]. The charged phospholipids rearrange themselves due to the incoming wave of electric field induced by the electroporator. As the rearrangement occurs, physical pores are created in the phospholipids bilayer acting as channels, allowing water and other materials to enter into the cell.

The effectiveness of electroporation is dependent on the size and purity of the plasmid DNA. A charged solution may cause the electrical field to weaken and diminish the rearrangement of the phospholipid bilayer, lowering the number or size of pores formed[20]. This would lower the amount of material flowing into the cell, reducing transfection efficiency. The DNA plasmid concentration affects transfection. A higher concentration of DNA plasmid results in a higher probability of materials entering the cell and hence higher transfection efficiency.

While no chemicals are added during electroporation, the procedure can cause cell death [21]. The formation of pores can be harmful to the cells. While pores are representative of

physical damage incurred, the cell membrane is generally capable of repairing itself, provided that the pore does not exceed a certain size. If the pores are too large, the cell would be incapable of repairing the rupture. This irreparable wound would cause the cell to become apoptotic.

Therefore, there are limits to how much transfection can occur before the cell sustains too much damage and goes through apoptosis. A careful optimization for each cell line is required to balance cell death and transfection rate [22]. The compromise in viability versus cell death after transfection is an inherent characteristic of electroporation. It is still used due to its ability to transfect a wide range of cells, including those that cannot be transfected chemically.

1.2.6 - Sonoporation

Ultrasound is a non-viral transfection methodology. Sonoporation, like electroporation, forms pores in the cell membrane to allow passage of drugs and DNA plasmids into the cells [23]. Sonoporation has the same advantages and disadvantages as electroporation. Sonoporation uses physical force to induce transfection in various types of cells, but is hindered by cell viability post-transfection [24]. Due to the penetrating property of sound waves, ultrasound transfection outperforms other techniques in terms of spatial localization.

Ultrasound, with the assistance of microbubbles, may create pores in the cell membrane and allow foreign material to enter into the cell, followed by membrane sealing [25]. Provided that the sound waves can reach to the specific tissue, sonoporation can take place. This means transfection can occur at desired locations inside the body. It has proven to be successful in delivering plasmids in mice and humans [26,27,28]. Furthermore, ultrasound is harmless to the body. The benefit of safety and localization gives sonoporation a competitive edge over other techniques in human clinical trials.

The purpose of this project is to investigate sonoporation and to establish techniques to overcome the inherent shortcomings of ultrasound induced transfection.

1.3 Ultrasound

Sound is a travelling wave of compressed molecules in a medium, capable of transferring mechanical energy [29]. The medium can be gas, solid or liquid. Sound can have various speeds, amplitudes and frequencies. The speed of sound is determined by the square root of Elastic modulus divided by density of medium [30]. Therefore, sound waves can travel much faster in solid than in air. Greater compression and rarefaction of the wave represents greater amplitude, which determines the loudness or the intensity of the sound wave [31], while frequency governs the tone. Human ears can hear only a small spectrum of frequency. The audible spectrum is called acoustic waves, while the frequency above the acoustic range is the ultrasound range, which as a lower limit of 20kHz [32].

1.3.1 - Current usage

Ultrasound is a common technology used in a variety of applications. It can be as common as household appliances such as cleaners and humidifiers. Sonar, which detects fishes and vessels, also uses ultrasound. Ultrasound has a wider range of applications in industries, such as plastic welding. In the biomedical field, there are two different branches of ultrasound usage.

The first is therapeutic applications. Physical therapy uses ultrasound as a device to treat patients. The ultrasound is used in diathermy, to warm up a patient's muscle or joint and physical therapist then guides the patient in therapeutic movements [33]. Generation of heat from ultrasound is caused by the movement of molecules in compression and rarefaction as it reacts to the transmitted ultrasound waves. Deposition of energy is greatest at the point where ultrasound waves are focused. The intensity for physical therapy is relatively low compared to lithotripsy. Lithotripsy is the use of ultrasound to break kidney or gall stones [34]. This process

uses focused high power ultrasound pulses to create shockwaves around the stones, which removes them without incision. It has been noted that tumours can be killed by similar focused high-energy ultrasound [35]. In addition, low-intensity pulsed ultrasound has been proven to facilitate growth of bones and teeth [36, 37]. There are further therapeutic applications of ultrasound in cosmetics.

The second important use of ultrasound is for imaging. The fundamental property for imaging is the propagation and reflection of ultrasound waves, just as bats use ultrasound to detect their surroundings [38]. Medical imaging is a miniature version of the sonar in marine technology. Ultrasound waves travel through the body and meet a change in tissue types. Different tissue types have different composition and thus, have a different density and elastic modulus. This gives rise to different characteristic acoustic impedance between tissues. Due to the change in acoustic impedance, some of the sound wave will be reflected backwards. By picking up these reflected fundamental and harmonic echoes, it is possible to create a one dimensional image of the tissue inside the body [39]. In the past, a simple two dimensional image can be taken by compilation of many one dimensional images. As technologies advance, compilation of many two dimensional images can form three dimensional images, as CT and MRI scans currently provide. Furthermore, ultrasound can be used to measure the flow of blood by utilizing the Doppler effect of sound [40]. The rate of flow could be measured by ultrasound through monitoring blood cells because the reflected frequency increases if the object is moving towards the probe and decreases as it moves away from the probe.

Most importantly, ultrasound generating machines are economical, portable, and safe. An ultrasound machine costs much less than other diagnostic imaging equipments such as CT, PET and MRI scanners. Therefore, hospitals can afford many ultrasound machines, with an associated decrease in wait time for diagnostic activities. These machines can be as compact as

a smart phone [41], which allows them to be used on immobile patients or moved outside of hospitals for diagnostic work. Most importantly, ultrasound is safe. It is approved by FDA (Food and Drug Administration of United States) and everyone can be imaged, including fetuses. In short, ultrasound imaging is commonly used and extensively developed in medical field.

1.3.2 - Properties

There are many attributes of ultrasound that can be controlled and adjusted in sonoporation. Each attribute represents a different property of sound waves. These attributes includes: frequency, intensity, duty cycle, repetition rate, beam profile, transmission and reflection [42].

There are a wide range of frequencies identified as ultrasound. The lower limit of ultrasound is 20kHz, which is the upper limit of hearing capability of human ears. The unit for frequency is Hertz, describing how many compression and rarefaction cycles occurs within a second. As frequency increases, the penetration power of ultrasound decreases [43]. From frequency ($f = \frac{1}{Period}$) and speed of wave (v), the wavelength (λ) can be calculated using the wave formula $\lambda = \frac{v}{f}$. Frequency is used to identify the characteristics of the ultrasound wave.

The attribute that plays a large role in determining energy deposition is intensity (I). Intensity is the amplitude of the sound wave. Intensity can be described by various units. In acoustic sound, decibel (dB) is a logarithmic scale used to compare the intensity of the sound waves. This scale compares to a reference standard and is not an actual unit. Intensity is also directly related to power, therefore, Watts per Meter Square can also be used $\frac{W}{m^2}$. This is the unit used in the experiments in this thesis. In the literature, there is another measurement of intensity, Pascal (Pa), which is also an international standard unit for pressure. This emphasizes the property of compression and rarefaction of sound waves. The two units, Watts/Meter^2 and

Pascal are very different in describing ultrasound wave. A direct conversion is impossible. Therefore, the unit in which ultrasound is described depends greatly on the instrument used to characterise the wave and the intent of the measurement. Watts/Meter^2 is measured by a power meter, while Pascals are measured by a hydrophone.

Other attributes that can affect energy deposition include repetition rate and duty cycle (DC). There are two types of waves that can be generated: continuous and pulsed. A continuous sound wave would have compression followed by rarefaction endlessly without a pause in between. If a continuous wave is in the acoustic range, one would hear a long unchanging tone. Pulsed sound waves have gaps between the trains of compression and rarefaction. A pulsed sound wave in the acoustic level could be described as short tones interspersed with silence. It may be difficult to hear the silence in between pulses because the length of these pauses is directly proportional to the repetition rate and the frequency. Repetition rate, also known as repetition frequency, defines how many pulses occur in a period. Duty cycle refers to the amount of time ultrasound is produced, or "on", in a given duration. Ten percent duty cycle would have one wave and nine silent intervals in ten cycles; a 20 percent duty cycle has one wave and four silent intervals in five cycles. A continuous wave can be described as an ultrasound with 100 percent duty cycle. For a ten percent duty cycle with 1MHz ultrasound and 100kHz repetition rate, the pulse would have a length of one microseconds (μs) and nine μs of silence. One μs is the duration restricted by frequency. The ten μs interval is varied by the repetition rate. Since energy is carried by sound waves' compression and rarefaction, energy is effectively diminished to a tenth of its value in continuous wave for ten percent duty cycle.

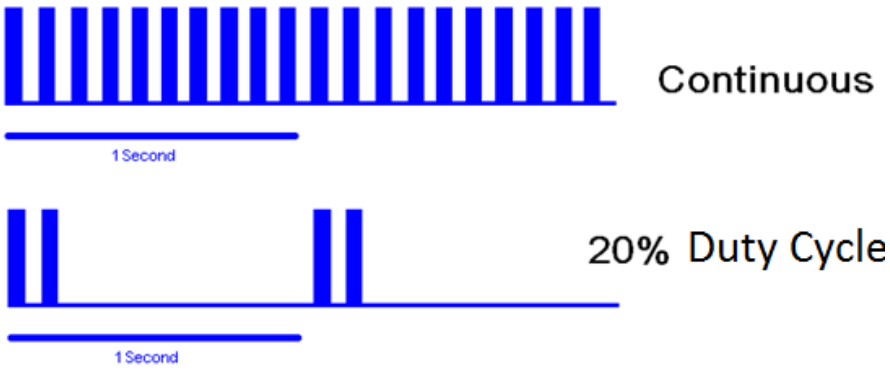

Figure 1.1 - The graphic representation of the difference in between Continuous ultrasound wave (Top) and Pulsed ultrasound wave with a 20% Duty cycle (Bottom)

The volume affected by ultrasound and the intensity received is determined by the beam profile. Beam profiles can be described as wide, narrow or focused. A wide beam profile would indicate the angle at which ultrasound leaves the transducer is large, spreading the ultrasound over a vast cross sectional area. A wide beam profile, compared to a narrow beam profile producing the same amount of energy, would have less energy deposited per unit area, but more volume is affected by ultrasound. A narrow beam profile is the opposite; the same energy produced by the transducer is spread out over a smaller amount of area, leading to more intense ultrasound per unit volume. A focused beam has the ultrasound strongest at a particular point away from the transducer where all the sound waves focus. The area affected is small but it has high intensity within that small dimension. One complication brought about by the various beam profiles is the measurement of intensity. Pascals can be further broken down to be Newton/Meter^2. The amount of pressure is dependent on the area the force is applied to. Therefore, to catch all the waves of a wide beam and to precisely measure the location of the focal point of the focused beam must be exercised with caution. The beam profile is strongly dependent on the transducer. Apart from the transducer, higher frequencies tend to have

narrower beam profiles than lower frequencies as they disperse less in unfocused ultrasound beams [44].

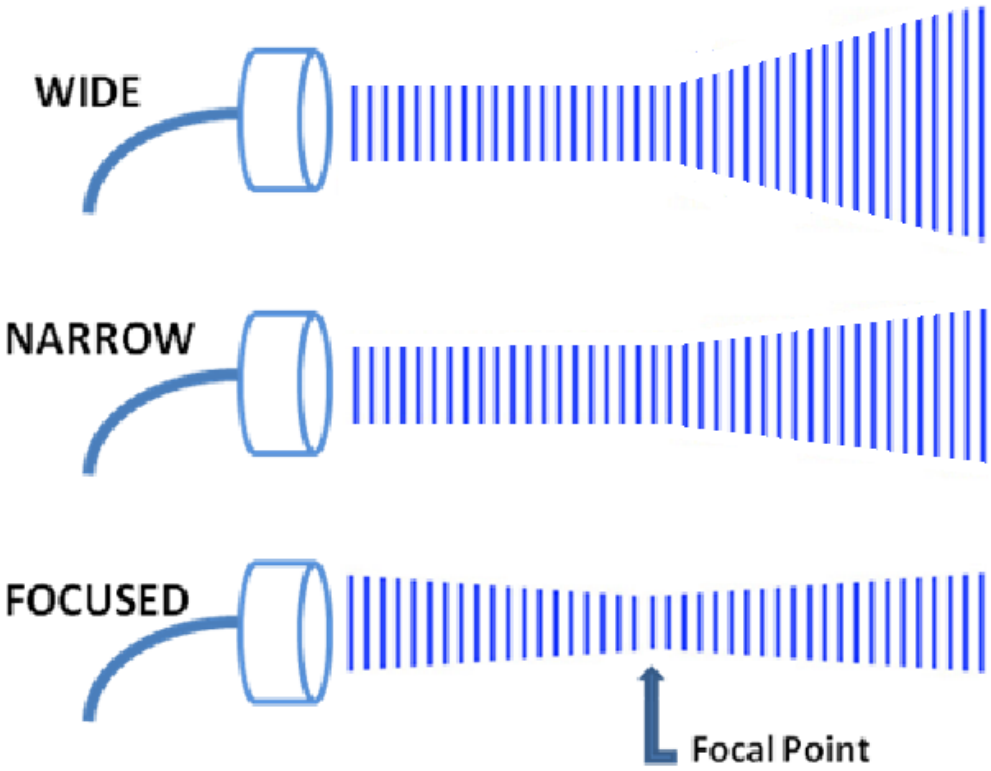

Figure 1.2 - The difference between ultrasound beam profile

Attenuation is one concern in designing an ultrasound system. Energy is lost through dissipation in the medium or by reflection of wave it as it travels through media with different acoustic impedance. This ultrasound phenomenon is called acoustic impedance mismatch; the bigger the mismatch, the stronger the reflection [45]. In ultrasound imaging, a gel is applied on the surface of the skin with the transducer submerged in the gel for better coupling between the transducer and the dish by pushing out all the air which could cause unwanted reflection and a decrease in energy transfer. Some researchers have minimized the number of surfaces ultrasound is required to pass through before reaching the cells for sonoporation by submerging the transducer and cells in water, which will uniformly transmit ultrasound, allowing the cells to

receive all the ultrasound waves produced by the transducer [46]. Reflection can cause an increase in the desired energy deposition. The desired ultrasound deposition comes only from the wave directly generated by the transducer. However, after passing through the cells not all intensity is absorbed, the remaining ultrasound can bounce back and hit the cells again, hence increasing the energy deposition. To prevent this, some researchers place an ultrasound absorbing pad behind the cells [46]. Attenuation, transmission and reflection all cause variance in the characteristics and energy deposition of ultrasound waves.

1.3.3 - Electronics and Ultrasound Generation

Most transducers on the market are using piezoelectric material. The piezoelectric effects suggest that certain materials are stressed mechanically after electricity is applied. These materials include special types of ceramic and crystals. An everyday example is quartz used in clocks and watches. The electrical stress causes the material to reversibly expand and contract. Every crystal has its distinct natural resonance frequency, which is determined by the material's speed of sound, elasticity, size and shape. The expansion and contraction of material creates the compressions and rarefactions required to generate ultrasound waves.

Piezoelectric material will stay stressed and will not relax after a charge is applied; therefore it is unable to oscillate in a static electric field. A constant input of electric pulses is necessary to drive oscillation in the piezoelectric material. The frequency of the electric pulse determines the oscillation frequency of the piezoelectric material. It is possible to drive piezoelectric material at a frequency other than its fundamental or harmonic frequencies, but the piezoelectric effect of the material will not be as dramatic, losing efficiency. The amplitude of the electric pulse determines the magnitude of contractions and expansion, thereby controlling the intensity of the ultrasound produced.

It is possible to reverse the piezoelectric effect to generate electricity by stretching and compressing the piezoelectric material mechanically or by coupling it to a source ultrasound. The efficiency of the electricity production is determined by the frequency and intensity of the incoming sound wave. Using this idea, piezoelectric sensors and indicators can be built.

1.4 Ultrasound Contrast Agent

1.4.1 - Origin

Ultrasound contrast agents are used in sonoporation experiments to increase pore opening. These contrast agents are used commercially in medical imaging, such as echocardiograms in the heart [47]. In sonography, these ultrasound contrast agents are used to increase the reflective property because of its large acoustic impedance differences with its homogeneous surroundings and its core. The increase of the reflective property translates to an increase to the resolution of the image [48].

Ultrasound contrast agents can be classified into five general types with various physical properties: free gas bubbles, encapsulated gas bubbles, colloidal suspensions, emulsions and aqueous solutions [49]. All of these share the same characteristics of having gaseous cores. These ultrasound contrast agents are injected intravenously into patients and circulate in the blood streams, including arteries, veins and the pulmonary capillary beds [50]. Without the contrast agent, blood vessels are imaged as black area because there is no reflection from the homogenous solution. With the aid of the ultrasound contrast agents, blood will reflect ultrasound waves back to the detector [47]. Advancements in imaging technologies are now able to detect harmonic and sub-harmonic reflections [51]. These reflections enhance the contrast of the blood vessel and make it possible to view them with clarity. This clarity provides information about the speed and flow of blood, organ perfusion and the detection of tumors [52, 53].

Ultrasound contrast agents are reflective because they are composed of gas bubbles, which have a very low density. When an ultrasound wave comes to the boundary of two different media, some of the waves will be reflected while the rest of the wave penetrates the next medium. The coefficient of reflection for a sound wave, $R = (\frac{Z_2 - Z_1}{Z_2 + Z_1})^2$, is dependent on the

impedance (Z) of the two media. The impedance is the product of the density of the medium and velocity of sound within that medium. The substantial difference in density and speed of sound between gases and liquids will lead to a large coefficient of reflection, which in turn, leads to effective imaging of the homogenous areas.

Any gas bubble is capable of increasing reflectivity of blood. A vigorously shaken saline buffer can act as a contrast agent in ultrasound imaging, but they may not be compatible with the body. Ideally, microbubbles should be non-toxic, capable of crossing the pulmonary capillary bed, and stable while being cost effective [54]. Therefore, gases in ultrasound contrast agents can be encapsulated by biocompatible materials such as proteins, lipids, or biopolymers to reduce their toxicity [55]. Their size usually ranges from one μm (micrometer) to seven μm [56]. There are many formulas and combinations of shells and gas cores to create ultrasound contrast reagents [57]. It is also possible to have shells labeled with a target marker, allowing them to attach to the surface of cells [58]. All of these differences contribute to the change in size, circulation time and reflective capability of these ultrasound contrast agent [59,60].

1.4.2 - Various Kinds

Ultrasound contrast agent can be self-prepared or commercially purchased [61]. The most common commercial brands for ultrasound contrast agents includes: Albeunex™, Levovist™, Definity™, SonoVue™, Sonazoid™ and Optison™ [48]. Optison™, under GE healthcare and approved by FDA, is widely used in literature for ultrasound-mediate transfection. However, Optison™ is not sold everywhere in the world, including Canada. In Canada, the only attainable ultrasound contrast agent is Definity™ by DuPont Pharmaceuticals and distributed by Lantheus Medical Imaging. Definity™ is currently used by hospitals in Canada and worldwide for ultrasound imaging.

It is also possible to make custom designed microbubbles as contrast agents. Perfluorocarbon- exposed sonicated dextrose albumin (PESDA) is a type of microbubble not branded under any companies. There are other albumin based microbubbles used in ultrasound imaging [62].

In a single type of ultrasound contrast agents, there are variations in sizes within the population of the microbubbles [63]. The deviation of bubble size's distribution curve may be different for various agents, but the same principle applies to all. The median size of the microbubble population is usually referred. The variation in sizes will cause a variety of oscillation responses under the influence of ultrasound.

1.4.3 - Cavitations

In sonoporation, the reflective capabilities of ultrasound contrast agents are not useful. However, the gas cores of the ultrasound contrast agents are able to facilitate the opening of cell membranes. Sound waves can be destructive and this destructive force is provided through the transfer of energy at the object's resonance frequency. When the sound wave's frequency matches the natural frequency of an object, resonance occurs. During resonance, energy can be transferred efficiently. When not in resonance, energy can still be transferred to objects through similar mechanisms, but it is much less efficient.

When a gas bubble is under the influence of ultrasound, energy from the sound wave can be transferred into the bubble. The energized bubbles then react in one of two ways: inertial cavitation and stable cavitation [64]. There are hypothesis on how both of these effects can increase transfection.

Inertial cavitation, also called transient cavitation, is generally thought as the main mechanism for opening pores on cell membrane [65]. In inertial cavitation, the energy

transferred to the microbubbles is greater than the microbubbles can handle. After rapid and rigorous expansion and compression of the bubble, gas cores can abruptly collapse [66]. This sudden collapse can lead to generation of high temperatures and high pressures [67]. The volume affected is highly localized [68]. Extreme conditions such as radicals and shock waves are also created [69]. Radicals are molecules or ions with unpaired electrons that are very reactive and can damage cell membranes and even affecting the genetic composition of the cell [70,71]. Shock waves are physical pressure waves similar to sound waves but travels faster and more energetic. These shock waves are capable of generating radiation as well as physically push and pull forces [72]. The cell membrane can be torn and damaged by force of the generated shock wave. Both radicals and shock waves are short lived; radicals will react with anything in its path and a shock wave's intensity decreases in proportion to the surface area of a sphere. Therefore, microbubbles need to be in the proximity of cells for cavitations to cause sonoporation.

Stable cavitation occurs when the energy transferred to the microbubbles are insufficient to generate inertial cavitations. The energy transferred to the microbubbles still causes the microbubbles to oscillate, expanding and shrinking periodically [73]. This oscillation leads to microstreaming, a small scale of acoustic streaming [74]. Acoustic streaming is the bulk non-periodic movement of fluid caused by ultrasound. The movement can be used to move solids, pump liquids and to cool operations. The ability of microstreaming to move molecules is the fundamental hypothesis of how stable cavitation aids transfection and increasing permeability [75].

Since size and composition determine the natural frequency of the microbubbles, various ultrasound contrast agents have different optimal ultrasound frequencies for stable or inertial cavitation. Moreover, variance in sizes within a population can cause both stable and inertial cavitation in the same sample of microbubbles. This suggests both cavitations work

simultaneously to increase permeability of the cell membrane and optimization for each ultrasound contrast agent is required.

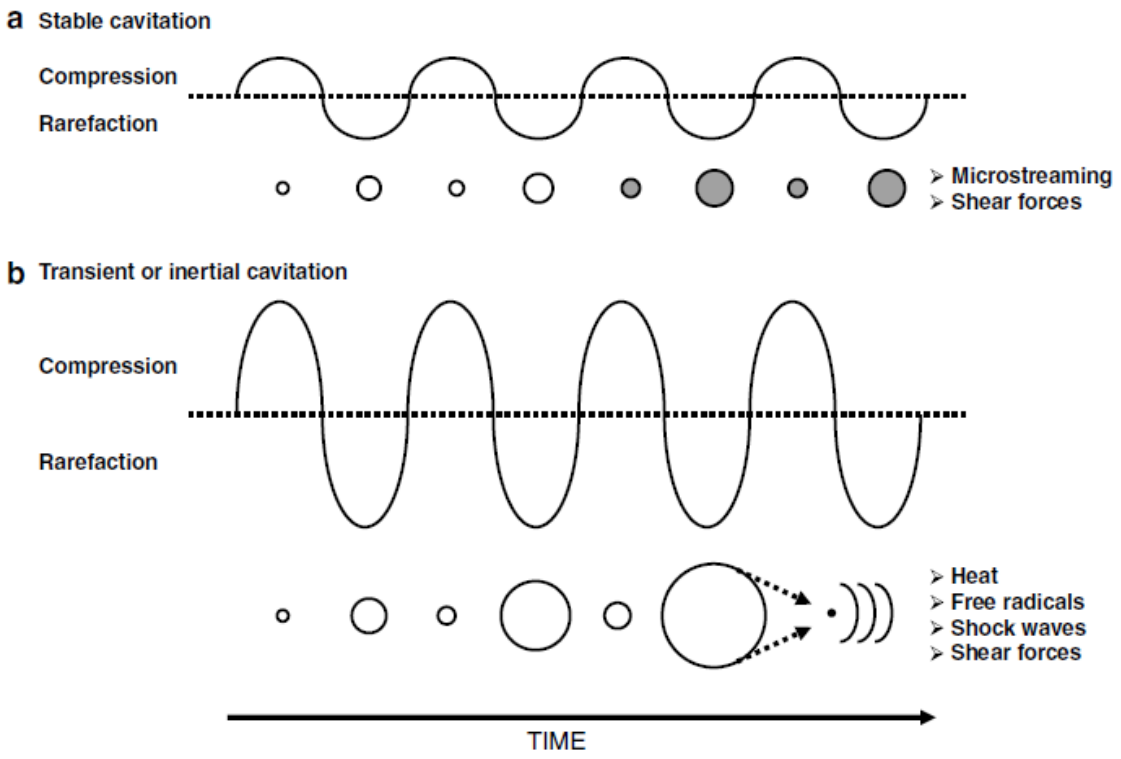

Figure 1.3 - Schematic illustrating of (a) Stable cavitation and (b) Inertial cavitation [64]

1.5 Cell Lines

1.5.1 -Stem Cells

Stem cell manipulation is one of the most popular topics in academia and plenty of resources have been placed on it [76]. Stem cells are very difficult to transfect and sensitive to foreign material. Safely transfecting stem cells with high efficiency, maintaining cell viability and preventing them from differentiating into body specific cells are some of the challenges.

The value of a stem cell is its ability to perpetually renew itself and to differentiate into many different types of cells [77]. A human totipotent stem cell can develop into any human cells, including extraembryonic tissues [78]. These are extremely hard to obtain. Totipotent stem cells are only attainable during the period between fertilization of an egg to blastocyst [79]. Once a blastocyst is formed, totipotent stem cells begin to specialize. Some become inner cell mass, while others become part of the outer trophoblast. The specialized totipotent stem cell can no longer sustain its ability to differentiate into all types of cells [79]. Embryonic stem cells, which are found in the inner cell mass of the blastocyst, lose the ability to differentiate into extraembryonic tissues [80]. Even though embryonic stem cell can develop into all human cells, it cannot form a fetus [81]. The embryonic stem cells are referred to as pluripotent due to its inability to form extraembryonic cells [82]. The pluripotent embryonic stem cells are the focus of stem cell research.

It is extremely difficult to find these pluripotent stem cells in adults. However, isolation of multipotent stem cells is achievable [83, 84]. Multipotent stem cells cannot differentiate into all types of cells in the body, but can differentiate into a range of related cells [85]. These multipotent stem cells do not directly mature into specific cells performing particular roles within tissue. Instead, they usually go through intermediate cells called progenitor cells. These progenitor cells are partially differentiated along a specific pathway and have lost their self-

renewal capacity [86]. For example, hematopoietic stem cells (HPSC) are capable of differentiating into various types of blood cells via various progenitor cells [87, 88]. This multipotency is still valuable because a therapy with the multipotent stem cell sources can affect all of the problematic cell types downstream for many future generations to come. This is the goal of stem cell therapy, leading to a long term effect of the therapy. While able to differentiate, stem cells are further capable of regenerating themselves and replenishing the number of stem cells after some of them have differentiated into progenitor cells. Since some somatic cells are not capable of regenerating themselves, such as red blood cells, treating these somatic cells via gene therapy will not have a long lasting effect. The cells that were cured go through apoptosis as it ages and will be replaced by the newly differentiated, uncured cells. Therefore, gene therapy is usually done upon multipotent stem cells.

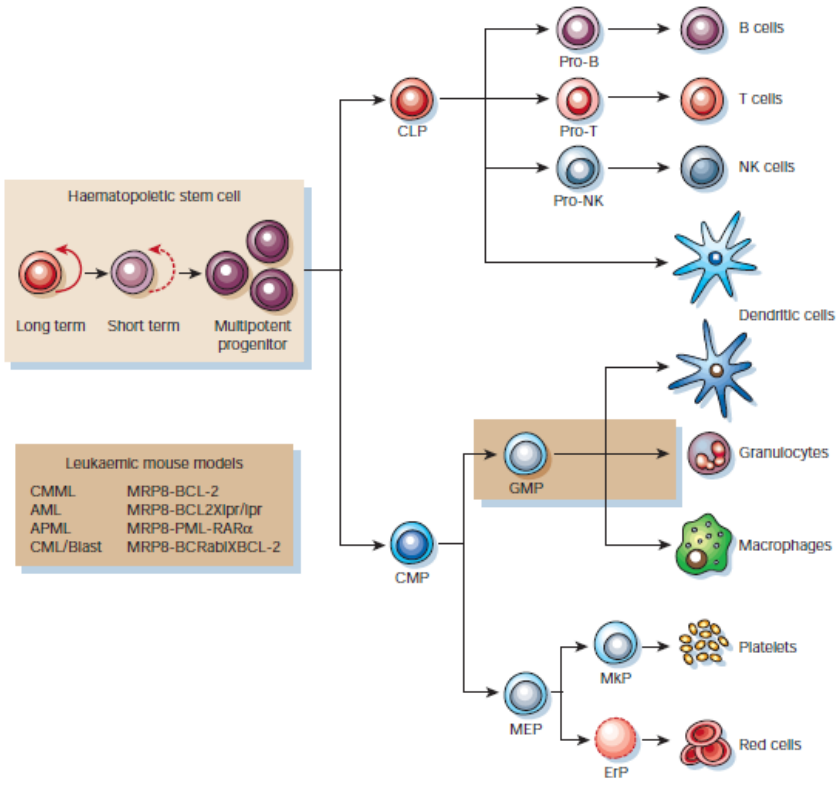

Figure 1.4 - Hematopoietic stem cell pathways [77]

Although multipotent stem cells are easier to obtain than pluripotent embryonic stem cells, they are still difficult to acquire in large quantities. The number of stem cells in the adult human body is very low. Furthermore, the extraction process for stem cells is inefficient. Therefore, it is not always possible to obtain a large amount of stem cell samples needed in research. Another way to obtain stem cells is from umbilical cords. Umbilical cords contain about two million hematopoietic stem cells. However, the quality of these stem cells varies from baby to baby. Moreover, it is not always possible to obtain fresh cord blood from newborns and it is expensive to purchase. This poses many difficulties technically and financially.

To have repeatable experimental results and constant availability, a stem cell model was used in research. The KG-1 cells a good model for hematopoietic stem cells [89], while MCF-7 is used to compare and contrast against the KG-1 cells.

1.5.2 - KG-1 Cells

KG-1 cells are purchased from ATCC (American Type Culture Collection). They were obtained originally from a 59 year old Caucasian male, who had erythroleukemia that evolved into acute myelogenous leukemia. These leukemic cells were located in the bone marrow and extracted through aspiration.

KG-1 is a suspension cell line, where the cells float in the medium during all phases of growth. KG-1 is a cell line which is not conducive to experimentation. They are not very compliant to experimental treatments and prove to be difficult to transfect by methods other than through viral vectors. Lipofectamine, the most commonly used chemical transfection agent, has proven to be ineffective against KG-1 cells [90]. KG-1 is a good representative of cell lines that are difficult to transfect and is used in experiments for this reason.

1.5.3 - MCF-7 Cells

In contrast to KG-1, the MCF-7 is an adhesion cell line. MCF-7 was originally isolated from the mammary gland of a 69 year old Caucasian female who had breast cancer. The cells form a monolayer on the bottom of the growth container before proliferating. MCF-7 cells are larger than that of KG-1. Unlike KG-1, these cells are easy to manipulate and can be readily transfected by various transfection reagents. For this reason, MCF-7 represents the cell lines which are easy to transfect.

1.6 Transfection Marker

When testing transfection methodology, it is necessary to measure the occurrence of transfection. There are many ways to test if transfection has taken place. It is possible to transfect a gene that codes for nothing and have that as transfection marker. Having the non-coding sequence of DNA inserted into the cell's genome would still be considered a successful transfection, since the DNA is inserted into the targeted cell. It is possible to confirm transfection by examining the targeted cell's genome. It is also possible to culture the targeted cells and examine later, if a successful stable transfection has taken place. Stable transfection requires the genes to be incorporated into the target cell's genome and allows the genes to pass on to the next generations. Hence, cells should still contain the non-coding sequence many generations after the transfection event. However, a gene can be expressed even if it is not in the genome, leading to what is referred to as transient transfection. The transiently transfected genes may then be lost in the next cell division. To select for stable transfection, cells can be cultured in a toxic environment, with a resistant gene incorporated into the foreign DNA. Only the cells with the new resistant gene inside their genome can survive and proliferate.

1.6.1 - Electrophoresis

It is also possible to test for transfection, both transient and stable, using a different approach. For an easy and generalized testing of the transfected DNA sequence, the sequence must be designed with care. The DNA sequence should contain at least two sections where restriction enzymes can cut. These restriction enzymes will cut the specifically designed DNA at a specific recognition nucleotide sequence known as restriction sites. After sections of the transfected sequence are spliced out, they can be separated and identified from the rest of the DNA genome of the targeted cells by electrophoresis (Southern blot). The amount of DNA

produced can be measured. The disadvantage of this protocol is the inability to distinguish whether the amount of DNA separated are results of high number of copies of gene inserted into the cells or a high number of cells being transfected. Small interfering RNA (siRNA) as transfection markers encounters similar problems.

SiRNA can suppress an existing gene function or induce an extra gene function temporarily. These up or down regulation of the translation of proteins can be detected by electrophoresis (Western blot). Similarly, the amount of proteins produce by the culture of cells transfected by siRNA can be measured, but it is impossible to determine if it was the result of a high number of copies of gene inserted into the cell or a high number of cells being transfected.

1.6.2 - Antibody

A functional gene that causes changes to the cell membrane can help determine between the two possible interpretations as experienced in electrophrosis, i.e. whether it was the high number of copies of gene inserted into the cell or the high number of transfected cells that indicated a positive result. Antibodies can mark whether the gene is expressed in the cell or not. Another set of antibodies will tag the primary antibody, which are attached on cell surface, with a fluorescent marker. Cells that express a surface protein coded by the transfected DNA sequence will have flouresecent antibody attached to them and can be counted using specialized machine, flow cytometry. This allows individual cells to be analyzed, which was impossible with electrophoresis. The disadvantage of this method is the need for antibodies, which can be costly.

1.6.3 - Plasmid Coding Fluorescent Protein

An economical method is to simply transfect genes that code for something that is directly measurable and allows successfully transfected cells to be counted. A Green Fluorescent Protein (GFP) is a protein that re-emits a green light after an ultraviolet or blue light is shone onto it. There are other proteins, such as luciferase, that re-emit an electromagnetic (EM) wave after a higher energy EM wave excites it. These proteins are coded by plasmid DNA. The plasmid must enter the cell, be transcribed into RNA and then translated by ribosomes into proteins. Similar to the surface proteins, the florescent protein coding plasmids do not have to be inserted into the genome, and can still be transcribed and translated transiently. The detection and measurement of the transfection of GFP can tell researchers how many cells were transfected and their expression level via fluorescent intensity.

Delivering genes for stable transfection requires that the DNA sequence be incorporated into the cell's genome. The amount of expression depends on the properties of the DNA sequence, such as the promoter region, and the physiology of the recipient cell. The DNA plasmid model employs more than one mechanisms, which has its advantages and disadvantages. To produce a protein, the DNA plasmid needs all three steps: sonoporation, transcription and translation, in order to have the expression analyzed. This emulates the process of transfection for gene therapy. However, since there are many variances from the transfected DNA and from cells, it may be difficult to directly compare one DNA sequence or cell line to another.

1.6.4 - Florescent Macromolecule

A different model can be used to eliminate the variance of the DNA sequence and cell physiology. If macromolecules can pass through the cell membrane with the help of a

transfection methodology, then it is assumed to be capable of allowing DNA to enter the same cell for transfection to occur. A bio-compatible fluorescent macromolecule replaces the DNA plasmid in this model. For example, fluorescein isothiocyanate (FITC) attached to Dextran is a good candidate for assessing the level of sonoporation [75, 91]. FITC is a small molecule that can be attached to other molecules and yet maintain its fluorescent capability. Dextran, a polysaccharide, comes in various sizes, allowing it to imitate the various lengths of DNA sequences. By using FITC-Dextran, transcription and translation is disregarded and the flow of material through the cell membrane is assessed. Cells that have FITC-Dextran inside are considered to be successfully sonoporated.

The FITC-Dextran model is used to explore the ability of ultrasound waves to open up cell membranes and allow foreign material to enter. Therefore, it is a model to emulate the delivery of drugs and DNA into the cell. On the other hand, DNA plasmid encoding fluorescent proteins is more suitable to emulate gene delivery and transfection of cell using ultrasound.

Chapter 2
Hypothesis and Objective

Advancements in nanotechnology have brought gene therapy and pharmaceuticals to a new era. However, these discoveries have yet to become mainstream therapies due to the immaturity of delivery methods. The established biological methods of delivering genetic materials into cells, such as: viral; chemical; and physical-based transfection methods, all have their short comings. For instance, the concern for viral transfection is its safety; chemical transfection is its cell dependent susceptibility; physical-based transfection is its high death rate. Much research efforts have been focused on exploring new means to transfect cells in a safe, robust and efficient manner. The goal of advancements in spatial selective delivery of genetic materials and cytotoxic drugs is to minimize costs and side effects.

Researchers believe that ultrasound poses a new possibility to a safe and reliable delivery method. Sonoporation, using ultrasound to physically increase cell membrane permeability, is a relative new and immature drugs and gene delivery method. Similar to other physical delivery methods, the greatest weakness of sonoporation is its high cell death rate. This thesis will explore and evaluate possible sonoporation plus microbubble techniques to minimize cell death.

Using a self-developed ultrasound device, SonaCell, and a commercial microbubble, Definity™, sonoporation protocols for the delivery of biomolecules and genes were developed on MCF-7 and KG-1 cells. MCF-7 and KG-1 cells were chosen for comparison because one is easy and the other is difficult to transfect cell lines. Suspension cells, especially KG-1 cells, are well known for its difficulty to be transfected using non-viral methods. Therefore, success in

demonstrating efficient transfection on KG-1 cells would prove the effectiveness of proposed transfection method.

In this thesis, comparison of traditional sonoporation method against four novel methods were made. The aim of these novel methods is to increase delivery efficiency while keeping cell death at an acceptable level. 1) In multiple sonoporation session treatment, cells will be treated with ultrasound treatment more than once. This should improve cell viability by eliciting cells' reparation mechanism to minimize damages from further sonoporation, as well as resetting the cell cycles of sample cell population to achieve higher efficiency. 2) Synergistic sonoporation with other chemical transfection reagents may enhance effects between the transfection agent and ultrasound, thereby increasing efficiency of transfection without increasing the cytoxicity of cell. 3) Sonoporation with a novel self-developed, chemically synthesized microbubble form carrier , which genes and drugs can be released at close distances to cells upon caviation, will increase the chance of cellular uptake and efficiency of delivery. 4) Proliferation ultrasound was proven to increase the regeneration and recovery of cells after sonoporation; performing sonoporation with proliferation ultrasound may also decrease cytoxicity of samples treated in harsh sonoporation conditions.

These new procedures were documented, performed, analyzed and evaluated for their feasibility and prospect for drug and or gene delivery.

Chapter 3
Material and Methods

3.1 - Materials

3.1.1 - Laboratory Machinery

- NUAIR class A2 Biohazard safety cabinet
- NUAIR autoflow CO2 air-Jacked Incubator
- Sorvall T1 Centifuge (Cat# 75002382, Thermal Scientific)
- Allegra 25R centrifuge (Beckman Coulter)
- Motic AE31 Inverted light microscope
- Laboratory counter (Cat#: 02-670-14, Fisher Scientific)
- Bright-line hemacytometer reichert 0.1mm deep
- Vortex mixer-touch (Cat#: 02215360, Fisher Scientific)
- Precision 180 series water bath (Cat#: 2823, Thermal Electron Corporation)
- Isotem Oven (Cat#: 13-247-751F, Fisher Scientific)
- Autoclave (Market force)
- 4°C fridge with -20°C Freezer
- -80°C Fridge (Thermal Electron Corporation)
- Confocal microscope (Carl Zeiss LSM510, Toronto, Canada)
- AJ100 electronic analytical balance (Mettler)
- FACs Caliber (Becton-Dickinson, San Jose, CA, USA)
- Excel UltraMax (Excel Technology Limited, Ontario, Canada)
- SonaCell System (IntelligentNano Inc.)
- Tektronics AFG 3251 Function Generator (Texas Instrument)

- 1.5MHz Transducer (American Piezo Ceramics International Limited)
- Adventurer Pro A114 Power Meter (Ohmic)
- ELx800 Absorbance Microplate Reader (BioTek)

3.1.2 - Labware

- Electronic Pipette-aid (Cat#: 1438678, Fisher Scientific)
- Costar pipettes
    - 5ml (Cat#: 4487, Corning)
    - 10ml (Cat#: 4488, Corning)
    - 25ml (Cat#: 4489, Corning)
- Air Displacement Single Channel Pipetters (Cat#: 21-377-328, Fisher Scientific)
- Pipetter tips
    - 100ul-1000ul (Cat#: 02-681-163, Fisher Scientific)
    - 1ul-200ul (Cat#: 07-707-504, Fisher Scientific)
    - 0.1ul-10ul (Cat#: 21-277-2A, Fisher Scientific)
- Ependorf microcentrifuge tubes
    - 2ml (Cat#: 508-GRD, Rose Scientific LTD)
    - 1.5ml (Cat#: 05-408-129, Fisher Scientific)
    - 0.5ml (Cat#: 05-408-120, Fisher Scientific)
- Disposable glass Pasteur pipets (Cat#: 13-678-20D, Fisher Scientific)
- Cell culture dish
    - 35mm (Cat#: 430165, Corning)
    - 1000mm (Cat#: 430167, Corning)
- Powder free nitrile gloves
    - Small (Cat#: 2705851, Fisher Scientific)

    - Medium (Cat#: 2705852, Fisher Scientific)
    - Large (Cat#: 2705853, Fisher Scientific)
- Laboratory parafilm 4inx 125feet roll (pechiney plastic packaging, Chicago, IL)
- Kimwipes(4.4x8.4in) (Kimberly-Clark professional)
- Cryopreservation vials (Cat#: 0334118E, Wheaton)
- Superfrost microscope slides (Cat#: 12-550-15, Fisher Scientific)
- Cover slips (Cat#: 12-548-B, Fisher Scientific)
- Rectangular canted neck cell culture flasks with vent cap
    - 25cm2 (Cat#: 430639, Corning)
    - 75cm2 (Cat#: 430641, Corning)
- Costar 96 well cell culture cluster (Cat#: 3596, Corning)
- Centrifuge tubes
    - 15mL (Cat#: 430055, Corning)
    - 50mL (Cat#: 430190, Corning)
- 5mL polystyrene round-bottom FACs tube, 12x75mm style (Cat# Falcon 352054, Beckon-Dickinson)

3.1.3 - Chemicals

- 0.25% trypsin-EDTA (Cat#: 25200, GIBCO)
- Antibiotics penicillin/streptomycin (Cat#: 15070, GIBCO)
- Cell culture medium
    - IMDM (Cat#: 12200, GIBCO)
    - DMEM (Cat#: 11965, GIBCO)
- Serum

    - Bovine growth serum (Cat#: SH30541.03, Hyclone)
    - Fetal bovine serum (Cat#: 12483, GIBCO)
- Phosphate buffered saline (1x) (Cat#: 14190, GIBCO)
- Fluorescein Isothiocyanate-dextran
- pDrive5-GFP-5 (Cat#: pdv5-gfp-5, InvivoGen)
- Zeocin™ Selction Reagent, liquid (Cat# R250-05, Invitrogen)
- 2% Bacto-Tryptone (Cat#: 211699, Beckman Dickson)
- 0.5% Yeast Extract (Cat#: 212710, Beckman Dickson)
- Sodium Chloride (Cat#: S3014, Sigma-Aldrich)
- Potassium Chloride (Cat#: P9541, Sigma-Aldrich)
- Magnesium Chloride (Cat#: M8266, Sigma-Aldrich)
- Magnesium Sulfate (Cat#: M2643, Sigma-Aldrich)
- Agar (Cat#: A7002, Sigma-Aldrich)
- 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (Cat#: 850365, Avanti Polar Lipid Inc.)
- Cremophor EL (Cat#: C5135, Sigma-Aldrich)
- Glycerol (Cat#: G5516, Sigma-Aldrich)
- Poly-L-Lysine 0.01% solution (Cat#: P4832, Sigma-Aldrich)
- Definity™ (Cat#: DIN 02243173, Lantheus medical Imaging)
- Trypan blue (Cat#: T6416-25g, Sigma-Aldrich)
- Paraformaldehyde, powder 95% (Cat#: 158127-500g, Sigma-Aldrich)
- Dimethy sulfoxide -DMSO (C_2H_6OS) (Cat#: 13P231-100, Fisher Scientific)
- 95% ethanol (Biochemistry Store, University of Alberta)
- Lipofectamine 2000 reagent (Cat#: 11668-027, Invitrogen)

- IBAfect (Cat#: PK-CT-2005-100, PromoKine)
- CellTiter 96 AQueous One Solution (Promega, Madison, WI)
- Thiazolyl Blue Tetra-zodium Bromide (Cat#: M5655-1G, Sigma-Aldrich)
- DNA Nuclear-ID™ Green Cell Analysis Kit (Cat# ENZ-51014-100, Enzo Life Science)

3.2 - Culturing Cells

3.2.1 Freezing and Thawing

Cells can be stored in liquid nitrogen for an extended period of time. They do not grow nor die when they are frozen in liquid nitrogen; all metabolism ceases when stored at -196°C. When required, the cells can be removed from the liquid nitrogen and thawed. These thawed cells can be cultured again. After culturing for a few generations, the cells will be stable and healthy for experiments. Reversibly, excessive cells from cell culturing can be collected and be frozen in liquid nitrogen for future use.

The extremely low temperature of liquid nitrogen requires special treatment during storage. The container for storing cells is specially made to withstand the extreme temperature. Cryogenic vials made from polypropylene are used for this particular purpose. To freeze the cells for future usage, $1x10^7$ cells were taken out of the culture. The cells were centrifuged at 1200 rpm for 5 minutes and the supernatant removed. The cells were resuspended in 600uL of freezing solution 1 and 600uL of freezing solution 2. The freezing solutions prevent cellular damage due to the freezing of the cellular components and solutions. The cells were placed in -80°C over night before transferring into a liquid nitrogen storage tank.

To thaw, the cryogenic vials were incubated in a water bath until the freezing solutions defrost. The thawed solutions were then moved into a 15mL centrifuge tube, where it was diluted by 9mL of culturing medium. The diluted solution was centrifuged at 1200 rpm for 5 minutes and the supernatant was removed. The cells were then resuspended in 10mL of serum-rich culturing medium and placed in a culturing container. The cells were checked the next day to determine their readiness for splitting.

The adherent and suspension cell lines have the same procedures for freezing and thawing.

3.2.2 - Cell Cultures

The two cell lines used were the MCF-7 (HTB-22) and KG-1 (CCL-246); both cell lines were obtained from ATCC (American Type Culture Conllection). The medium, serum and antibiotics used in culturing these cell lines are purchased from Invitrogen or Thermo Fisher Scientific.

The KG-1 cells' basal culturing medium is Iscove's Modified Dulbecco's Medium (IMDM) with L-Glutamine and phenol red. The phenol red is used to indicate the pH of the medium; a change in color suggests a change in pH which requires a change of culturing medium. 100mL of Bovine Growth Serum (BGS) and 5ml of Penicillin/Streptomycin (P/S) and were added to 400mL of IMDM to make a nutrient-rich culturing medium: 20% BGS + 1% P/S in IMDM.

MCF-7 cell's basal culturing medium is the Dulbecco's Modified Eagle Medium (DMEM) with high D-glucose concentration, L-glutamine, and HEPES buffers. Instead of BGS, the MCF-7 cell line prefers Fetal Bovine Serum (FBS). In 450mL of DMEM, 50mL of FBS and 5mL of Penicillin and Streptomycin were added to make the nutrient-rich culturing medium: 10% FBS + 1% P/S in DMEM.

The suspension KG-1 cells are cultured in a volume state. The medium containing cells is placed inside a cell culture flask from Corning. The maximum volume capacity of the flask is 60mL. The rectangular flask is laid on its largest side while it is inside the incubator from NUAIR for cellular propagation. The incubator electronically regulates the temperature and carbon dioxide level at 37°C and 5% CO_2. Humidity is not regulated, but tubs of water are placed inside the incubator to allow water to be evaporated into the air inside the incubator. The MCF-7 cells are propagated in the same conditions as the KG-1: humid, 37°C air with 5% CO_2. This regulation is to emulate the conditions of a human body. However, MCF-7 are adherent cells which grow while attached to the bottom of the culturing container. It would be more convenient to grow

MCF-7 on a large culturing dish, so that pipette tips can reach every part of the container when harvesting the cells. In this case, a large circular culturing dish with the dimension of 100mm^2 x 10mm from Corning was used.

3.2.3 - Harvesting and Splitting Cells

KG-1 cells are easier to harvest and split. KG-1 cells are already in cell suspension, therefore, cells can be taken out of their culturing container and be counted directly. Cells were placed into a 50mL centrifuge tube and were spun at 1500 rpm for 5 minutes in the centrifuge. The cells were then resuspended and diluted in nutrient-rich medium to an appropriate cell density for culturing or experimentation. The renewal of medium is done weekly to ensure the abundance of nutrients. During the week, every 2-3 days, the cells were counted; extra cells were removed and nutrient-rich medium was added to the culture to maintain the cell density between 2x10^5 to 1x10^6 per mL.

There are a few more steps in harvesting or splitting MCF-7 cells compared to KG-1. MCF-7 needs to be detached from the bottom of the culturing dish before they can be counted and split. 0.25% Trypsin was used to detach cells. Trypsin is a protein specialized in degradation other proteins, it is commonly found in digestive system. Trypsin digests the protein that MCF-7 secretes to hold onto the bottom of the culturing container. Depending on the size of container, different amount of trypsin is required. In the 100mm^2 Corning cell culture dish, 4mL of 0.25% Trypsin should be used. The amount of trypsin used should be sufficient to cover the bottom of the culture dish. The dish with trypsin was placed in incubator for 5 minutes. The culture dish was then checked under the microscope for detachment. The adhered MCF-7 cells have jagged corner when growing on the culture dish; detached cells will be afloat and have a round

spherical shape. The culturing container can be placed back into incubator for another 5 minutes if all cells have not detached.

To ensure all the cells were detached and collected, same amount of medium was added to the culture dish. The mixture of medium and trypsin was nutrient-rich pipetted up and down with force, washing the bottom and the edge of the culturing dish to detach the remaining cells. The cells were then placed in a centrifuge tube and centrifuged at 1200 rpm for 5 minutes. The medium and trypsin supernatant was removed. This removes trypsin from the cell as these protease can cause damages if the cells remain incubated in it for extended periods. 10mL of new medium is added back into the tube to resuspend the cells. The resuspended solution can then be used for cell counting, experiment or create a new passage of MCF-7.

After counting, adequate amount of cells will be moved to a new centrifuge tube. Nutrient-rich medium was then added to the cell to dilute the cell density. Number of cells and volumes of nutrient-rich medium are different for various cell lines and culturing containers. For the $100mm^2$ culturing dish, medium was diluted to the final volume of 10mL. The solution was mixed thoroughly by pipetting up and down before moving into the cell culturing dish. Splitting of MCF-7 should be done every two to three days to ensure the cells are not over populated and have sufficient nutrient to grow. Cells must not exceed confluence of 100%; i.e. all area is filled with cells. Full confluence will change the growth rate and metabolism of the cells.

3.2.4 - Cell Counting

A hemocytometer and a microscope are required to count cells. A hemocytometer consists of a chamber, which allows cells to be placed into it. The chamber has a mirror like bottom with a nine square grid. The area of each square is one milometer (mm) by one mm, and the height of the chamber is 0.1 mm. Therefore, each square represents the volume of $0.1mm^3$

and be able to contain 0.1 uL of medium. By counting the number cells in the square, and since the volume is set, it is possible to deduce the concentration or density of cells in the solution. From the concentration, we can calculate the number of cells we have in the cell culture by finding the volume of the cell culture. The size of the chamber and the size of cells are too small to be viewable by the naked eye; a microscope is necessary to assist in the viewing and counting of these cells.

There are other techniques developed to assist the process of cell counting to make the number more accurate. One possible problem is to have too many cells in 0.1 uL of cell culture, which makes it difficult to view and keep track to avoid double counting. By diluting the 0.1uL of the cell culture, it is possible to lower the number of cells to be counted per square. Diluting the cell culture will effectively changes the concentration of samples to the cell culture, hence, it is necessary to take that ratio into account. Less than 40 but greater than 15 cells inside each square is an optimal range for counting or else the statistical error margin would be too great for an accurate count. If the cell culture's concentration was too low, the cell culture was centrifuged at 1200rpm for 5 minutes, and were resuspended in a smaller volume of medium. This will effectively increase the concentration and in turn increases the number of cells in 0.1uL of cell culture.

Another technique to minimize statistical error is to count more squares and determine the average the count of cells. Averaging over many samples eliminates the counts that are outliers, where the number is not representative of the concentration in the medium. As more samples taken into the average, it helps to improve the accuracy of the average cell concentration. Therefore, it is recommended that the four corner squares of the nine squares grid on the hemocytometer be counted and have the concentration average out by four samples.

Accuracy of the count is further improved if the alive cells are counted. Using the trypan blue exclusion test, it is possible to distinguish living and dead cells. Trypan blue is a chemical compound that does not enter into the cell if the cell membrane is healthy, which suggest the cells is alive. However, trypan blue can enter cells that are dead. The dead cells will appear to be dark blue and shriveled up, while the healthy cells will be bright and round. Trypan blue needs to be diluted to 0.4% before it can be added to the cell culture medium. Since adding trypan blue will dilute the cell concentration, it is commonly used to dilute cells as previously mentioned. The trypan blue exclusion test adds functionality to cell counting by indicating the ratio of dead to alive cells in the culture. This ratio of dead and living cells suggests the relative health of the culture, and whether the cells can be used for experimentation.

To count cells, 10uL of suspended cell culture was removed out and placed into a small centrifuge tube. An adequate amount of 0.4% trypan blue was added to the small centrifuge tube for dilution and the trypan blue exclusion test. The centrifuge tube was then vortexed for few second and pipetted up-and-down a few times to mix the trypan blue and the cells together thoroughly. 10uL of the trypan blue mixed cell culture is pipetted into hemocytometer's chamber carefully without the formation of air bubble inside the chamber. The loaded hemocytometer was placed under the microscope. The four corner boxes were counted; alive cells and dead cells both being counted. The numbers are then converted into a cell culture concentration. An example with 10uL of cell culture mixed with 10uL of trypan blue, the dilution factor is two, and 152 cells counted in the four (4) squares of 0.1uL in volume:

$$152 \div 4 \times 2 \div 10000 = 76 \times 10^4 \; cells/mL$$

3.3 Cell Marker

In the experiments , GFP plasmid and FITC-Dextran were used as transfection and sonoporation markers. GFP serves as suitable transfection marker because it simulates gene delivery. Transfection is considered successful when genes are incorporated into the genome and are expressed. In order for GFP to act as a transfection marker, the plasmid would have to enter the cells, and be translated and transcribed into protein products. FITC-Dextran is a chemical marker that can be detected without going through cellular metabolism. It is suitable to detect sonoporation and to measure the permeability of cells via ultrasound mediated delivery.

3.3.1 - D.N.A. Plasmid: Green Fluorescent Protein

The GFP used in the experiment was bought from InvivoGen. The plasmid, pDrive5-GFP-5, a circular bacterial plasmid with 3502 base pairs which contains a red-shifted variant of the jellyfish GFP gene producing a protein that absorbs blue light (major peak at 480nm) and emits green light (major peak at 505nm). The pDrive5-GFP offers 10 different levels of expression with different promoters. The one used in the experiment has the hCMV-hCMV-HTLV promoter, which is at the 5th level of expression.

hCMV-hCMV-HTLV promoter consists of human CMV enhancer and minimal promoter which allows the GFP on plasmid to be transcribed into mRNA. The mRNA would have a HTLV 5' untranslated region (5' UTR) at the beginning of the mRNA codings for GFP. 5' UTR play a role in regulating gene expression. The tail of GFP coding mRNA is linked to a codon of Simian Virus 40 polyadenylation signal (SV40 pAn). This SV40 pAn gene enables efficient cleavage and polyadenylation, leading to a high level of steady-state mRNA.

Other than promoters and polyadenylation signal genes, there are genes which help in

culturing and selection. The plasmid is grown and proliferated in E. coli. To selectively allow bacteria that are transfected with the plasmid to grow and suppress the growth of bacteria that are not transfected, an antibiotic resistant gene was inserted into the plasmid along the promoters and GFP gene. The antibiotic gene in the plasmid gives resistance to is Zeonsin™. Therefore, with Zeonsin™ selection agent, bought from Invitrogen, was added to the growing medium and only bacteria which have the pDrive5-GFP plasmid will grow, leading to higher plasmid yield.

3.3.2 - Growing GFP Plasmid Competent E.Coli

The plasmid is transfected into E. coli for expansion. There are two medium required to grow E. coli, Luria-Bertani (LB) and Super Optimal Broth (SOB). LB has a lower nutrient level than SOB, but is used in pre-culturing E. coli. LB is made of 10g of 2% bacto-tryptone, 5g of 0.5% yeast extract and 10g of NaCl (sodium chloride) mixed into one litre of deionized water. SOB uses 20g of bacto-tryptone, 5g of 0.5% yeast extract, 2mL of 5M (molar) of NaCl, 2.5 of 1M KCl (potassium chloride), 10mL of 1M $MgCl_2$ (magnesium chloride) and 10mL of 1M $MgSO_4$ (magnesium sulfate) mixed into one litre of deionized water. Both of these medium are autoclaved, at a high pressure to sterilize the medium.

Competent E. coli was first plated on agar. Agar was made from one litre of LB medium mixed with 15g of agar, and then autoclaved. Before the solution fully cools and settles into a gel form, Zeonsin™ was added and the solution was poured onto petri dish. The air bubbles of the solution in the petri dish were removed. The solution was allowed to dry and solidify in the hood for an hour. Using a flamed (sterilized) wire loop, an inoculum of bacteria was streaked across a corner of the agar plate. After re-sterilization with a flame, a second streak was made at another corner of the agar plate by passing through the first streak with a wire loop. A third

streak is made similarly by passing through the second streak. The plate is then incubated at 37°C and grown for a day for colonies to develop.

From that agar plate, a single colony was picked and placed into 5mL of LB medium with 2uL of Zeonsin™ anti-biotic in a 50mL Corning centrifuge tube for liquid pre-culture. The centrifuge tube's top was wrappped with a breathable cloth and was then secured in a temperature regulated shaker, 37°C and 300rpm. The liquid pre-culture was shook for 8 hours. The agar plate with E. coli colonies can be sealed with parafilm and placed upside-down in 4°C and stored for a month; other colonies can be used as incoculum another liquid pre-culture during that period.

After 8 hours of growth in LB medium, 200uL of the pre-culture was added to 100mL of SOB medium with 5uL of zeonsin in a 250mL flask. The flask was placed back into the temperature regulated shaker, 37°C and 300rpm, for another 16 hours; more than one flasks can be shaken concurrently. The left over from the liquid pre-culture can be stored in 15% glycerol (150uL of glycerol is added to 850uL of E. coli liquid pre-culture) at -70°C. the glycerol stock can be stored for many years and used as inoculum bacteria for agar plating in the future.

Large amount of E. coli with the pDrive5-GFP plasmid was grown in the SOB solution and the plasmid requires extraction before they were used for transfection experiments. The plasmid separation kit used to extract the plasmid is by QIAGEN. The Plasmid Maxi Kits was chosen because it is suitable for the usual yield of the E. coli liquid-culture. The instructions from the manual of the plasmid separation kit were followed. After many steps requiring high speed centrifugation with temperature controlled with the Beckman coulter allegra 25R centrifuge, plasmid DNA wasseparated and resuspended in deionized water. The DNA solution was then measured for concentration, in nano-grams per micro-litre (ng/uL).

To ensure GFP plasmid was produced, electrophoresis could be done. However, an actual transfection using lipofectamine on MCF-7 was used as standard to determine if the pDrive5-GFP plasmid was produce and functional.

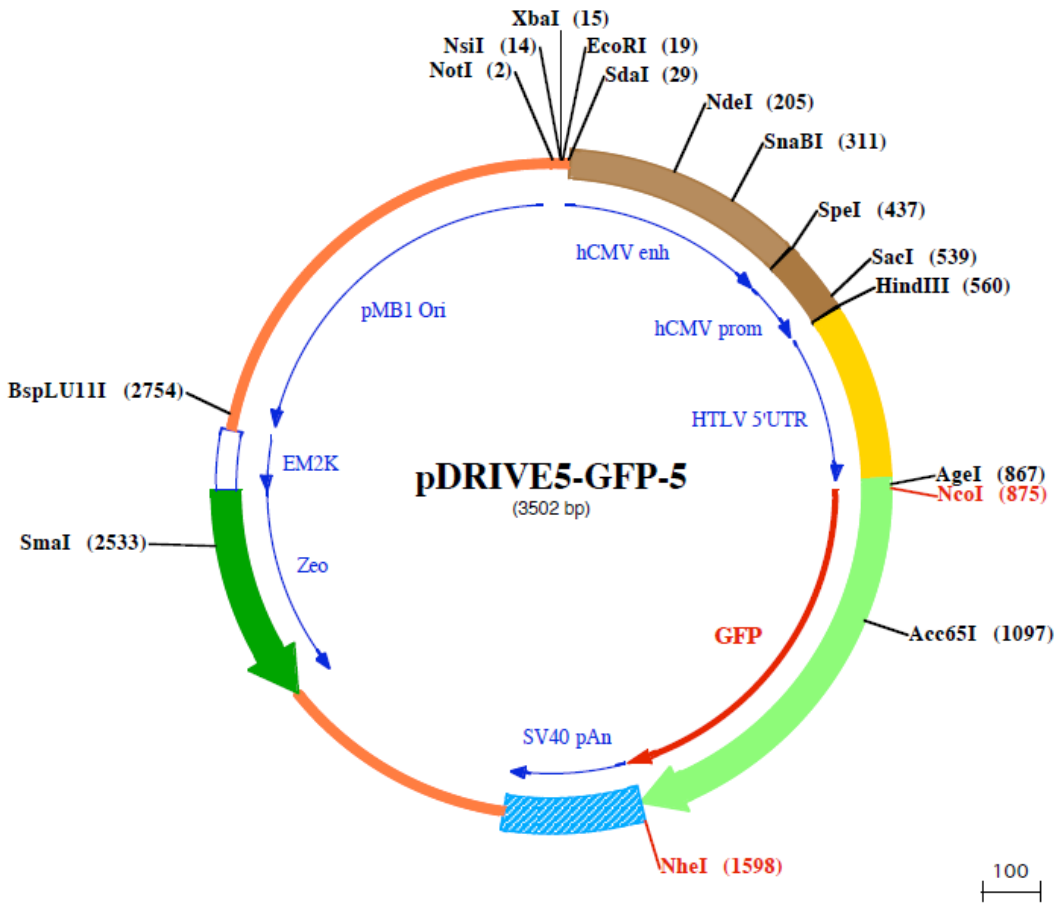

Figure 3.1 - A picture representation of the GFP plasmid used in experiments: pDrive5-GFP-5 from InvivoGen

3.3.3 - FITC-Dextran

FITC-Dextran was used in sonoporation experiments. FITC (flourescein Isothiocyanate) is a small fluorescent compound which absorbs blue lights and re-emits green light. The fluorescent molecule is attached to Dextran, a polysaccharide. The size of Dextran can

varies; it is made of braches of glucan chains. The size of Dextran chosen for experiment has an average molecular weight of 500,000; for every mole of Dextran, 0.003 - 0.020 mole of FITC is present. Powdered FITC-Dextran was bought from Sigma-Aldrich in Oakville, Ontario, Canada and was dissolved in PBS before it is used in experiment.

3.4 - Plating of Cells

The two cell lines used in the experiment were MCF-7 and KG-1. They are adherent and suspension cells respectively. However, it is possible to treat them while they are not in their native culturing form; MCF-7 can be treated while it is suspended, KG-1 can be forced to attached to the bottom of the plate.

3.4.1 - Attached Adherent Cell

For experiments on MCF-7 which were attached to the bottom of culturing dish, the preparation should be done one day before the actual experiment. The day before the experiment, MCF-7 cells were detached, counted and diluted to desire concentration. For sonoporation experiment, the cell density would be $1.5x10^5$ cells in 350uL of nutrient-rich medium samples; for chemical transfection samples $5x10^5$ cell in 350uL of nutrient-rich medium. A tray from the temperature and CO_2 regulated incubator was taken out and cleaned with alcohol (70% ethanol). It was then placed into the fume hood. 35mm x 10mm treated polystyrene culturing dishes from Corning were placed on the incubator tray. 350uL of medium containing cells were carefully placed in the centre of the dish, without any of the medium touching the side wall. The medium should form a large bead if placed correctly. The lids were then placed back onto the dishes. The tray was returned into the incubator with care not to tilt and cause the medium to move in the dishes. The cells were allowed to be cultured overnight until the next day. The dishes were viewed under the microscope the next day; the MCF-7 cells should have attached to the middle of the dish and not anywhere else.

3.4.2 - Suspended Cells

MCF-7 cells can also be treated while in suspension. They were treated similary to KG-1 cells in suspension. Sonoporation and chemical transfection can be performed on suspended cells. The cells were first detached, counted and diluted to $3x10^5$ cells in 350uL of nutrient-rich medium for MCF-7 cells or $7.5x10^5$ cells in 350uL of nutrient-rich medium for KG-1 cells. 350uL of medium containing cells were transferred to a 1.5mL microcentrifuge vial from Ependorf, where GFP plasmid, microbubbles, and/or transfection reagents were added. The solutions inside the vial was pipetted up-and-down for mixing . All of the solution was placed on a dish flatly mounted onto the ultrasound transducer. The cells were placed in the middle of the dish, forming a bead of medium of the 35mm culturing dishes. The cells were ready for sonoporation experiment.

3.4.3 - Attached Suspension Cells

However, for better transfection using chemical transfection reagent, KG-1 cells were forced to attach to the bottom of the culturing dish with chemicals. This attachment was facilitated with poly-L-lysine 0.01% solution from Sigma. Poly-L-lysine was used as primer; 700uL of poly-L-lysine was added to each dish and soaked for 30 minutes. The dishes were frequently tapped to ensure poly-L-lysine was covering all parts of the dish. After 30 minutes, poly-L-lysine was removed and the dishes were carefully rinsed with 1mL of PBS. Meanwhile, KG-1 was centrifuged at 1500rpm for 5 minutes and medium was removed. KG-1 cells were diluted to $7.5x10^5$ cells per mL of PBS. 1mL of the KG-1 containing PBS was added to the dishes primed with poly-L-lysine. After allowing the KG-1 cells to attach to the bottom of the dishes for one hour, the dishes were checked under the microscope for attachments. The dishes were shook lightly while viewed under the microscope to determine if the cells were attached. If the cells

did not slosh along with the PBS, the cells were attached and PBS was carefully removed. The cells should be treated immediately with transfection reagents because poly-L-lysine does not permanently attach KG-1 cells onto the bottom of the dish, as KG-1 cells detach as time passes by. Furthermore, the dish should be treated with care, as rigorous shaking or forceful pipetting will also cause KG-1 cells to detach from the bottom.

3.5 - Lipoplexes and Polyplexes

There were three chemical transfection reagents used in the experiments. They are polyethlyenimine (PEI), Lipofectamine 2000 and IBAfect. PEI was prepare by another lab; Lipofectamine 2000 was purchased from Invitrogen; IBAfect was purchased from PromoKine. The preparation and usage between the three reagents were similar.

3.5.1 - Polyethlyenimine (PEI)

For PEI, 2ug of GPF was mixed into 100uL of basal medium in one vial, while 5ug of PEI was mixed into 100uL of basal medium in another vial. The two vials were then mixed together and placed in the 37°C, 5% CO_2 incubator for 30 minutes. The PEI-DNA can then be added to the cells for transfection. The PEI-DNA complex needs at least 5 hours of incubation at 37°C, 5% CO_2 along with cells for effective transfection. After 5 hours, the medium of the cells was carefully removed in MCF-7 cells and 1mL of nutrient-rich medium was added back to the cells. For KG-1 cells, 1mL of nutrient-rich medium was added to dilute the tranfection reagents. Both cell lines were grown for another 24 hours before collected for results.

3.5.2 - Lipofectamine 2000

For Lipofectamine 2000, 4ug of GFP plasmid was placed in 250uL of basal medium. In a separate vial, 10uL of Lipofectamine 2000 was added to 250uL of basal medium. After waiting for 5 minutes, the solutions in the two vials were mixed together. The newly mixed solution was placed at room temperature for 20 minutes. 500uL of Lipofectamine 2000-DNA plasmid complex was added into 350uL of cells contained in basal medium. The mixture was then transferred to the 35mm cell culturing dish. The cells were then placed in 37°C and 5% CO_2 incubator for 24 hours before collected for analysis or for further cultured in the 37°C, 5% CO_2 incubator by

removing the solution (through centrifugation for KG-1 cells) and adding 1mL of nutrient-rich medium.

3.5.3 - IBAfect

The third chemical transfection reagents tested in the experiments was IBAfect. Similarly, 4ug of GFP plasmid was placed in 100uL of basal medium in one vial, and 9uL of IBAfect in 100uL of basal medium in another vial. The two vial was mixed together by pipetting up-and-down without waiting. The mixture was incubated at 37°C and 5% CO_2 for 20 minutes before 200uL of the solution was moved into 350uL of basal medium with cells. The cells with IBAfect-DNA plasmid complex was placed in the temperature and CO_2 regulated incubator, 37°C and 5% CO_2. After 5 hours of incubation, the medium of the cells was carefully removed in MCF-7 cells and 1mL of nutrient-rich medium was added back to the cells. For KG-1 cells, 1mL of nutrient-rich medium was added to dilute the tranfection reagents. Both cell lines were grown for another 24 hours before taken for results.

3.6 - Hydrated Phospholipid Microbubbles

Microbubbles, such as ultrasound contrast reagents are used in sonoporation. They help to facilitate pore formation on cell membrane through cavitation after ultrasound waves energize them. Microbubbles are nanometer sized bubbles with gaseous core. The can be purchase or self prepared. Definity™, similar to Optison™, was bought commercially. In the experiments self-developed microbubbles was used to compare and contrast the results of Definity™, the primary microbubbles used in this thesis.

The self-developed microbubble consist of a phospholipid shell and atmospheric air as its gas core. The formulation can be varied, but the procedure to synthesize these hydrated microbubbles is consistent. The phospholipid consist of a hydropillic headgroup and hydrophobic fatty acid chains. The phospholipid used for the sonoporation experiments is the 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) purchased from Avanti Polar Lipid. The phospholipids forms a more stable microbubble by adding surfactants. The surfactant used in producing microbubbles for experiment is Cremophor EL and Glyerol from Sigma-Aldrich. The ratio between phospholipids and surfactant can be varied to adjust the stability of the microbubbles. The microbubbles used in the experiments have DSPC to Cremphor EL ratio of 10:1.

3.6.1 - Synthesis Procedure

Correct amount of DSPC and Cremophor EL was measured and placed inside a round bottom flask from Pyrex. The ratio between DSPC and Cremophor EL was 10:1. One mL of Chloroform from Sigma-Aldrich was added to the round bottom flask to dissolve the chemicals. The solution was placed inside the 60°C Isotem Oven from Fisher Scientific for 15 minutes. The round bottom flask was placed in a rotary evaporator for 30 minutes, until chloroform had

evaporated. A PBS and glycerol mixture was prepare at a ratio of one to one; the mixture was used to dissolve the powder in the round bottom flask after evaporation until the concentration of DSPC was five mg per mL of mixture. The round bottom flask was then placed in a 60°C water bath for two hours while stirred at 700rpm. After the solution was cooled, it was stored in -20°C fridge until experiments was performed.

Quality check of the microbubble produced by the solution was performed for every batch. The activated microbubble solution was placed under the microscope to check for bubble sizes' uniformity. Batches which failed to possess the desired size or not uniformly distribute were discarded. Microbubbles were also tested in 1MHz, 20% Duty Cycle, 0.5 W/cm^2 and 100Hz repetition rate to ensure the microbubble will disintegrate.

3.7 - Sonoporation

There are three parts to prepare in the protocol of sonoporation: cells plating, GFP and Definity preparation and ultrasound application.

3.7.1 - Green Fluorescent Protein

Cell plating was explained in previous section. GFP plasmid was added to the cells on the day of the experiment. GFP was grown, separated, its weight and concentration determined as described in previous section. The amount of GFP (in ug) was calculated and converted to volume (in uL) through the concentration. The calculated amount was pipetted into the medium containing suspension cells. Medium from the adherent MCF-7 cells samples was replaced by 700uL of basal medium, and GFP was directly added onto the medium. The plasmid was incubated at 37°C and 5% CO_2 with the adherent cells for 15 minutes before adding microbubbles into the solution. For suspended MCF-7 or KG-1 cells, the calculated amount of GFP was added to the 350uL of cells in basal medium and the samples were ready for sonoporation experiments.

3.7.2 - Microbubbles

Microbubbles needs to be prepared. The microbubbles used in the experiments were provided by the ultrasound contrast agent Definity™ (injectable Lipid-encapsulated Perfluoropropane Microbubbles) from Lantheus Medical Imaging and by self-developed phospholipid microbubbles. Both types of the microbubbles need to be activated through rigorous shaking by a machine provided by Lantheus Medical Imaging. The self-developed phospholipid coated microbubbles were used as a comparision with Definity™.

3.7.2.1 - Definity Microbubbles

One package of Definity™ comes with four vials of 1.5mL of inactive Definity solution. The inactive solution is transparent and has a water-like appearance. It was design such that the whole amount of 1.5mL should be shook together, however, since our experiment does not required the whole bottle of Definity™, some of the inactive solution was stored in a sterilized 1.5mL of micro centrifuge vial from Ependorf at 4°C. After removing the lid of the Definity™, a needle and syringe was used to puncture the rubber seal and some of the inactive Definity™ solution was removed for storage, leaving at least 500uL of Definity™ in the original vial. 500uL is the minimum amount to be shook; from experience, the higher the volume of Definity™ shook, the longer the Definity™ stays active. The lid was placed back on top of the vial and parafilm was used to wrap around the lid and seal it. The bottle was then mounted onto the activation machine and was shook for 45 second. The activated Definity™ will become milky white. The active Definity was taken out of the original packaging vial and placed into a small sterilized vial.

3.7.2.2 - Phospholipid Microbubbles

The previously prepared self-developed hydrated microbubbles solution was thawed. 100uL of the solution was placed in a 1.5 mL Ependorf microcentrifuge tube with 100uL of PBS. The microcentrifuge tube was placed into the Definity™ activation machine for 45 seconds. The vigorously shaken solution was activated and was ready to be used as microbubbles as Definity™.

3.7.3 - Mounting Cell Culture Dishes

Just before the cells were sonicated, microbubbles was added and mixed with the medium through pipetting up-and-down. Microbubbles was added directly and mixed into the

350uL of medium containing the suspended cell. For adherent cells, the dishes with cells attached in the middle were mounted on transducer directly. However, for suspended cells, a new dish was mounted first before cells were placed into the dish. An ultrasound gel was used to hold the dishes on top of transducer as well as to minimize the attenuation of ultrasound buy preventing impedance mismatch which cause the ultrasound waves to bounce back before reaching the samples. A small amount of gel was placed on the transducer, then a dish was positioned on the gel and was pressed down gently, removing all the air bubbles that might have been trapped in the gel. The removal of air bubble ensures good transmittance of ultrasound from the transducer to the dish. The ultrasound transducer should be position directly below the centre of the dish.

The adherent MCF-7 can be treated right away with ultrasound after the sample dishes were directly mounted on the transducer as the dishes already contains cells, GFP plasmid and microbubbles. The suspension cells were loaded onto the mounted dish before sonication ,carefully placed in the centre of the dish, directly above the transducer, before ultrasound can be applied.

3.7.4 - Ultrasound Machine

3.7.4.1 - Exel UltraMax

There were two system of ultrasound used in the experiment. The first system is an ultrasound box which is commercially available and originally designed for physical therapy. The Excel UltraMax box can produce ultrasound in two different frequencies, 1MHz and 3MHz. Is capable to output intensity ranges from 0.1W/cm^2 to 2W/cm^2 at both frequencies. The transducer the box used has a surface area of 5cm^2. The size is not critical as the cells are placed in the middle, and the medium bead is smaller than the size of the transducer. Therefore, watts

per centimeter square was used to record the ultrasound's characteristics. The Excel UltraMax is also capable to choose between duty cycle of 10%, 20%, 50% or continuous. The duty cycle interval is 10 ms, or 100Hz pulse repetition; i.e. if the duty cycle was set to ten percent, ultrasound pulses would be 1ms in duration which repeats every 10ms.

3.7.4.2 - SonaCell

The second system was a self-built ultrasound system, which was used in other projects previously. The system is based on the SonaCell ultrasound box sold under IntellgentNano. It consists of two circuit boards, a function generator and a transducer. The function generator Tektronics AFG 3251 was bought from Texas Instrument. The electric output from the function generator was sent to the two self-built circuit boards, which in turn outputs amplified signal to a transducer bought from American Piezo Ceramics International Limited. The function generator's purpose is to shapes the characteristics of the ultrasound wave created by the transducer through generating electrical functions. These functions control the shape of the pulse and duty cycle. The function generator also exerts its influence on frequency, but does not control the output ultrasound's intensity.

Frequency and intensity are determined by transducer. Inside the transducer is a piezoelectric crystal, which under alternating current, will expand and relax. This expansion and relaxation is the mechanism for generating the pressure wave in ultrasound. These crystal can be driven at any frequencies, but they will not be as efficient as they would be if they were driven at their natural resonance frequency. This natural resonance frequency of a piezoelectric crystal determines the optimal output frequency of the transducer. The function generator should be driving the transducer at its natural frequency to effectively generate ultrasound.

The circuit boards of our self-built system function to control the repetition rate and to boost the amplitude of the sound wave. The borad modify the pulsed input from the function generator to produce a 1Hz pulse repetition rate; i.e. if DC is 20 percent, then it will produce a 200ms pulse train. The electrical signal coming from the function generator itself does not have enough power to drive a transducer, therefore, an external power source is required. The self-built circuit board takes the electrical signal from the function generator and increase its amplitude by using electricity from a direct current power supply connected to a wall outlet. By doing this, the board controls the intensity of the electronic wave sent to the transducer, hence, controlling the intensity of the ultrasound wave produced. There is a knob included on the circuit which tunes the power to the desired output. The intensity is calibrated against the Adventurer Pro A114 power meter from Ohmic. The power meter outputs in watts or grams.

The transducers used for the system has natural frequencies of 1.5MHz, with surface area of 3 cm^2. Therefore, the readings in watts were divide by 3 cm^2, the surface area of the transducer, to find the intensity of ultrasound the system is generating. The measurement by the power meter of the ultrasound intensity output is available in grams; from grams, it is possible to evaluate the system in Pascals.

$$Pa = \frac{kg}{m * s^2} = \frac{1000 * grams}{gravity}$$

3.8 - Confocal Microscope

The confocal microscope is situated at the Cell Imaging facility in Cross Cancer institute. The LSM510 confocal microscope is made by Zeiss. There is a computer program which fully controls the confocal microscope's options and operations. After placing the sample in the holder, location where confocal microscope should image was selected and parameter such as depth and quality was set on the computer program. The confocal microscope will automatically take an image of the selected area using a laser. The confocal images can be complied into a three-dimensional animation.

Confocal microscope images are used to see the morphology of the cells and to confirm delivery or transfection visually. However, this method cannot accurately account for the number of cells exhibiting fluorescent nor the fluorescent intensity because the area selected to be image is small and is statistically biased. Therefore, this method was used in auxiliary to confirm the success of the experiment.

3.8.1 - Samples Preparation

To create insightful confocal microscope samples, few preparation steps were performed on cell samples. First, cells were attached to a cover-slip of the microscope slide. For suspension KG-1 cells, the cover-slip was place in the 35mm culture dish, with 500uL of poly-L-lysine placed over the cover-slip. After 30 minutes, poly-L-lysine was removed. The coverslip was gently wahsed with PBS and 1mL of KG-1 cells in PBS was added onto the coverslip. The concentration of KG-1 in PBS should be around $1x10^5$ cells per mL. The KG-1 should have attached to the bottom of the cover-slip after an hour of incubation at room temperature. For MCF-7 cells, they were replated on the microscope slide after the experiment.

The immobilized cells were fixed with 1mL of 4% paraformaldyde after PBS or medium was removed. The samples were placed in 4°C fridge for 24 hours. The samples were gently washed with 1mL of PBS twice the next day after fixation. Rhodamine phalloidin, which stains the F-actin of the cell red, was diluted with PBS + 0.5% BSA (bovine serum albumin, used to block unspecific binding of dye with cell membrane) to 5uL per mL and 1mL of the dye was added to each sample. The staining was done in an unlit fume hood for 30 minutes. The dye was removed and was gently washed twice with PBS.

A drop of mounting solution containing DAPI was placed on a new microscope slide. DAPI (4',6-diamidino-2-phenylindole) is a blue colored stain that is used to stain the nucleus. The previously prepared microscope cover-slip with cells was taken out of the 35mm culturing dish and placed onto the mounting solution of the microscope slide with the cell attached side facing down and touching the mounting solution. The microscope slide was sealed with nail polish.

3.9 - Flow Cytometry

In contrast to confocal microscope, flow cytometry is an objective but non-visual method to check for transfection. The machine used for flow cytometry was the FACs Calibers from BD Bioscience, therefore FACs refers to flow cytometry. FACs is a function in flow cytometry, FACs is the acronym for Fluorescence-Activated Cell Sorting. The FACs Calibers is able to sort cells into different containers base on a cell's fluorescent level. To achieve this, every cell have to be monitored. The FACs machine is capable of recording the side scattering (SSC), forward scattering (FSC), various fluorescent color and its intensity of every cell passin gthrough the system. The measurement was done through a complicated system of mirrors, filters, lasers and photdetector.

Cells ins PBS are sucked up by the FACs machine, the solution passes through a tiny tube, allowing only a single cell to pass thought at a time. At that narrow bottle neck, the measurements are taken. Through many optical lens and mirrors, laser beams arrive at the bottle neck and pass through the cell. The transmitted, scattered and re-emitted light are project onto detectors. The data will then be stored by a computer program, Cell Quest. The program is also capable of showing these data on graphs. SSC and FSC are shown on a two dimensional scatter plot graphs, which give information on cell morphology, hence suggest whether the population's relatively health (Figure 3.2a) The fluorescent level is displayed in histograms, intensity on the x-axis and number of cells in each intensity bin on the y-axis, thereby telling the fluorescent level of the population (Figure 3.2b). Cell Quest also allows user to select a group of cells and sole display those cell's fluorescent level. This selection allows user to analyze the fluorescent intensity of the healthy cells only.

There are no absolute values in FACs, all data are relative. Therefore, controls are necessary for each experiment for comparison. Untreated cells were used as controls for FACs;

they are prepared the same way as other samples.

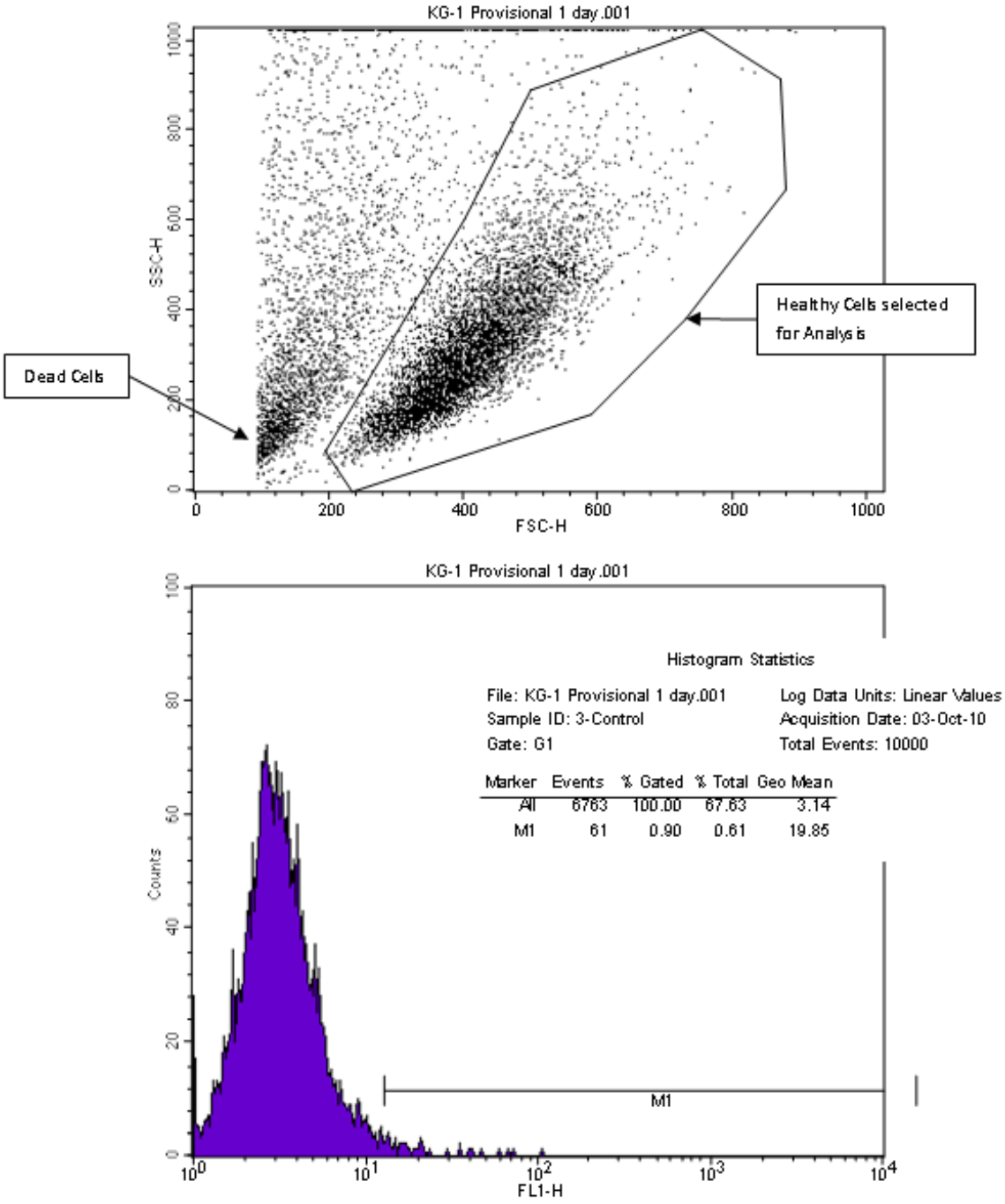

Figure 3.2 - An example of flow cytometry results from a control group of KG-1 cells. Top graph (a) shows morphological data of samples. Bottom graph (b) shows florescent intensity of the selected cells from top graph.

3.9.1 - Sample Preparation for GFP Transfection

Cells were collected in an unlit fumehood for flow cytometry analysis at the end of the experiments. For MCF-7, medium in the 35mm dishes was removed and were incubated with 700uL of trypsin for 5 minutes in 37°C, 5% CO_2. After checking the cells under the micrscope and ensured they have detached, 700uL of nutrient-rich medium was added to the trypsin and the mixture was pipetted around the dish to wash cells off the surfaces. The cells were then moved to a FACs tube from BD. For KG-1 cells, the cells were directly moved into FACs tubes. The dishes were washed with 1mL of PBS, and the PBS was also moved into the FACs tube. The cells in the FACs tube were spun for five minute at 1500rpm. The supernatant was removed and the cells were resuspended in 1mL of PBS. The cells were spun again at 1500rpm for 5 minutes for another wash. Most of the supernatant was removed, leaving behind about 200uL behind. Another 200uL of PBS was added to the tube, and the cells were resuspended in the solution. At this point, cells are ready to be read by the FACs machine for analysis. However, it is also possible to fix it and analyze it at a later time. To fix the cells, 100uL of 2% paraformaldehyde was added to the FACs tube and was mixed with the cell containing solution by vortexing. The samples can then be stored in 4°C fridge for a week before analysis. The freshly prepared or fixed samples were vortexed and placed in the FACs machine for analysis.

3.9.2 - Sample Preparation for FITC-Dextran Delivery

Similarly, adherent MCF-7 were detached with 700uL of trypsin and five minute in 37°C, 5% CO_2 incubation. 700uL of nutrient-rich medium was added to the detached MCF-7 and the mixture was pipetted around the dish to wash off cells from the surface. The cells were moved into FACs tubes. For KG-1 cells, the cells were directly moved into FACs tubes. The dishes were washed with 1mL of PBS; the PBS was also moved into the FACs tube. The FACs tubes were spun

for five minutes at 1500rpm. The supernatant was removed and the cells were resuspended in one mL of PBS. The cells were vortexed for 30 seconds and then spun again at 1500rpm for 5 minutes for another wash. The cells were washed again with one mL of PBS, 30 seconds of vortex followed by a five minute centrifugation at 1500rpm for a third time. This helps remove excesses FITC-Dextran in solution and to increase reliability of the FACs results. The supernatant from the third wash was removed, leaving behind 200uL of solution. 200uL of PBS was added to the samples. Before placing the freshly prepared samples in FACs machine for analysis, 50uL of filtered 0.4% trypan blue was added to quench the FITC-Dextran that remained on the cell membrane; allowing only the FITC-Dextran fluorescent inside the cells to be detected and measured.

3.9.3 - Cell Cycle Analysis

Another technique which analyze cell cycle can also be done by flow cytometry. Cells in different cell cycle will have different signature of DNA contents. By exploiting this fact, it is possible to analyze the amount of cells in each phase of the cell cycle within the population. The DNA Nuclear-ID™ Green Cell Cycle Analysis Kit from Enzo Life Sciences was used. This kit allows alive cells to be stained and viewed in FACs samples. However, the resolution was better with cells fixed first, and then stained before analyzing with FACs.

The kit provides Nocodazole, a drug that arrest cells at G2/M phase of the cell cycle by by depolymerizing microtubules necessary to pull chromosomes apart. The Nocodazole is used to create a control, where the sample of cells are all at G2/M phase. This control can be used to gauge the resolution of the technique, as well as a to help setting the parameters of the FACs machine. 0.1 µg/mL concentration of Nocodzole in growing medium was used to incubate the cells for 24 hours before they were collected, fixed and stained.

Samples are fixed with ethanol, because ethanol permeabilize the cells after fixing them. The samples were collected and washed as they would be fixed using 2% paraformaldehyde as previously described. However, instead of adding 100uL of 2% paraformaldehyde, 4.5mL of 70% ethanol at -20°C was added slowly into each FACs tube. The sample was placed in -20°C fridge for at least 30 minutes before it was stained. The ethanol fixed samples can be stored for months before staining and analysis.

To stain the ethanol-fixed samples, the cells were spun at 1500rpm for 5 mintues and the alcohol was removed. The samples was washed with 4.5mL of 1X assay buffer, prepared by diluting the 10X assay buffer provided in the kit by 9 parts of PBS. After vortexing for 30 seconds, the cells were spun again at 1500rpm and 5 minutes, the assay buffer was removed. 1uL of Nuclear-ID™ Green Cell Cycle Detection Reagent provided by the kit was diluted in 500uL of PBS. The solution was transferred onto the samples. The samples were incubated for 30 minutes at room temperature before analyzed through flow cytometry.

3.10 - Cell Proliferation Assay

Flow cytometry is an excellent technique to view the transfection or delivery efficiency by examining each cell individually and measure its fluorescent level. It is also possible for flow cytometry to measure the physical shape of a cell through light scattering. It is possible to tell the health of a cell by observing its shape, however, this is not accurate. A skewed morphology does not directly translate into dead cells. Therefore, flow cytometry results were not used to determine the percentage of cells in the population that were alive.

The assays used to determine cell viability are MTT and MTS assay. Both MTT and MTS assays use chemicals to determine the relative health of the cells population in samples. The chemical compound of MTT and MTS assay are taken in by cells and are actively reduced by the cell's reducing enzymes. The chemcial processsing would case the MTT and MTS assays' compounds to change colour. The cells were then lysed and allow the reduced dyes to be analyzed.

MTT assay uses the chemical Thiazolyl Blue Tetra-zodium Bromide from Sigma The chemical formula for MTS assay is 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and is purchased from CellTiter, CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Both of these dyes originally appears to be yellow, but changes to purple after being reduced by the cells. MTT assay is used for adherent cells, such as MCF-7 cells; while MTS assay is mainly used for suspension cell lines, such as the KG-1 cells. This difference calls for two different the protocols.

3.10.1 - MTT Assay

MTT assay was used for adherent MCF-7. MTT powder was dissolved in PBS at the concentration of five ug/mL. The powder does not dissolve easily, hence it was vortexed until no

`perceptions can be seen in the solution. Medium of the MCF-7 samples in 35mm dishes was removed.100uL of the freshly prepared solution and 900uL of nutrient-rich medium were added to the samples. The samples were incubated in a five percent CO_2 and 37°C incubator for approximately 1 hour. The samples were checked for colour change during incubation. If no apparent change in color at the bottom of the dish was observed, all samples were placed back into the incubator until color change can be observed. The incubation time should not exceed 4 hours.

The solution containing the MTT dye can be removed after enough dyes have been reduced by the cells. The cells were lysed open to allow the dyes to flow out of the cell. 700uL of Dimethyl sulfoxide (DMSO), a polar solvent, was used to break apart the cells' membrane and dissolve the reduced MTT dye. Dishes with DMSO were placed back into the incubator for 15 minutes to ensure all cells were lysed. After 15 minutes, the 200uL of the solution was placed into a well of a 96 well cell culture cluster (96 well plates) from Costar (catalogue number - 3596). The samples were triplicated; 3 wells was filled, each with 200uL of DMSO solution from the same sample. A triplicate of only DMSO solution was filled and compared against as background. The 96 well plate was then ready to be placed into a optical density (OD) reader.

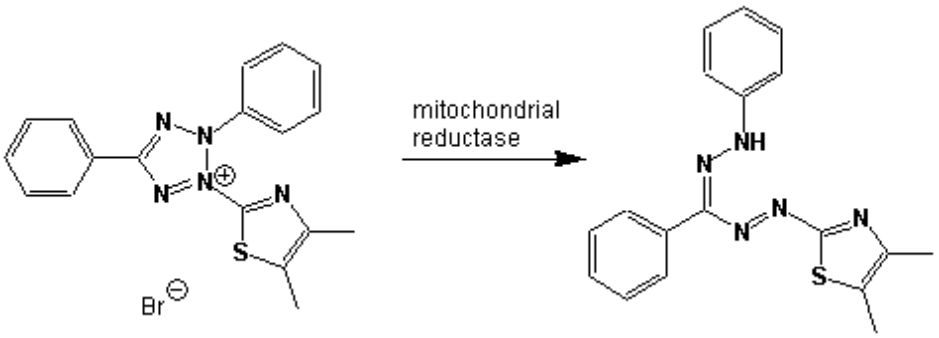

Figure 3.3 - MTT reduction scheme [92]

3.10.2 - MTS Assay

Samples of KG-1 cells were transfrred into a FACs tube. The cells were spun at 1500rpm for five minutes and supernatant was carefully removed, leaving 200uL behind. 200uL of nutrient-rich medium was added to the left over supernatant and the cells were resuspended in the solution. 100uL of the cell containing solution was then moved to a 96 well plates. Each sample was triplicated. A triplicate of only nutrient-rich medium was added to the 96 well plates as background. 20uL of the MTS solution from the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was added to the wells containing samples. The plate was placed in a 5% CO_2 and 37°C incubator for 1 hour. The samples were checked for colour change during incubation. If no apparent change in color at the bottom of the dish was observed, all samples were placed back into the incubator until color change can be observed. The 96 well plate is ready to be placed in an OD reader if a change of color in the well was observed.

3.10.3 - Optical Density Analysis

For both MTT and MTS assay, they were read with the ELx800 Absorbance Microplate Reader from BioTek. Gen5 is a computer program which controls the reader. The 96 well plate containing MTT or MTS samples was the place in the reader and the OD of each well was read for absorption at 490nm for MTS samples and 550nm for MTT samples. Gen5 will record the OD readings of the reader and transfer them onto a Microsoft Excel spreadsheet. The OD reading should be between 0.4 to two for simple analysis. The data from the triplicate of each sample will be reviewed and outlier was dropped; the other OD values of samples will be adjusted for background by subtracting the OD of the background. The adjusted OD values of the same sample were averaged using Excel and was used to compare against each other.

The comparison is done with respect to sample controls, where no experimental treatment was done upon. The controls are assume to have perfect viability. OD difference

between samples and control will imply a change in viability.Their ratio of their OD difference directly reflects the percentage of the cells alive, because OD and cell numbers are linearly correlated between OD reading from 0.4 to two.

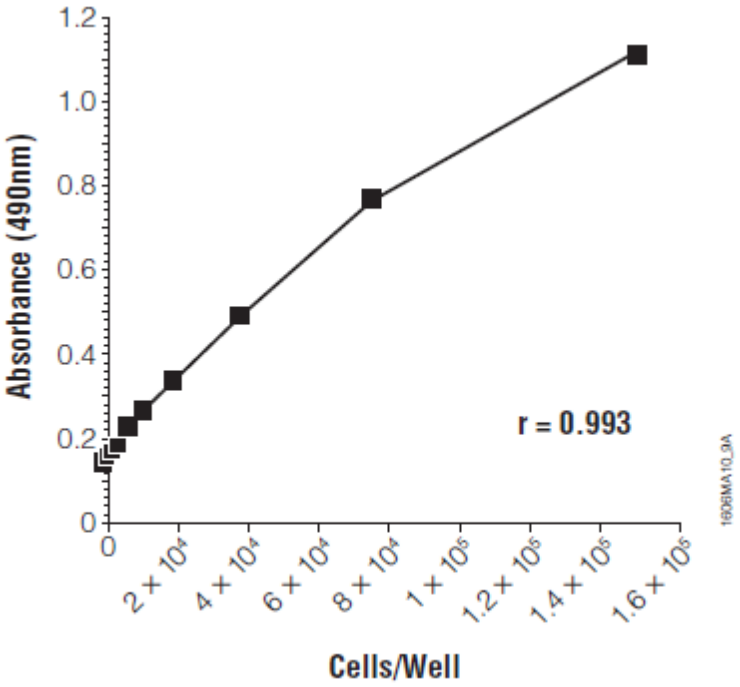

Figure 3.4 - MTS assay absorbance as a fuction of the number of B9 hybridoma cell. Graph taken from CellTiter Instruction Manual

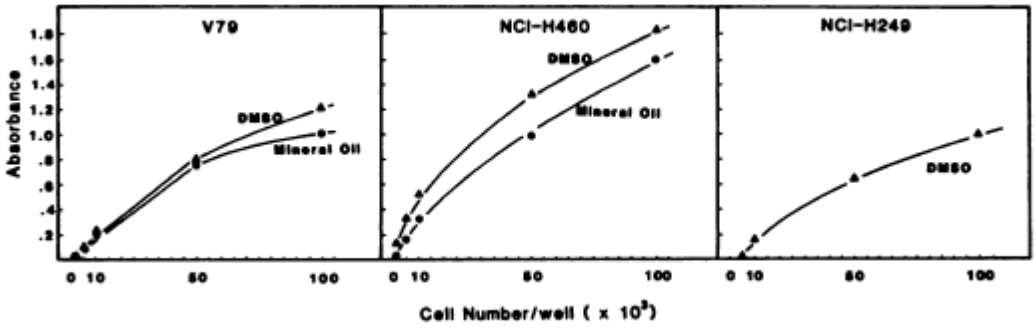

Figure 3.5 - MTT assay absorbance as a function of cell number with various cell line [93]

Chapter 4
Results and Analysis

4.1 - Setting Up Sonoporation Apparatus

Four parameters of sonoporation need to be explored and optimized for each cell line. They are as follows: concentration of microbubbles, number of cells in solution, concentration of GFP/FITC-Dextran and ultrasound characteristics. Furthermore, there are different parameters which make up ultrasound characteristics, such as intensity, frequency, duration, duty cycle and pulse repetition. In the beginning, each parameter was isolated and tested to establish basal parameters where sonoporation could occur.

4.1.1 - Energy and Definity Concentration's Impact on Cell Viability

To efficiently explore and improve sonoporation methods, it is necessary to establish the suitable range of parameters where sonoporation can occur, while maintaining a high cell survival rate. It was established that sonoporation causes cell death and the number of cell deaths is proportional to ultrasound intensity. The first test of this thesis was to confirm if such a trend can be repeated with the apparatus that would be used for all experiments.

There are many parameters of ultrasound that can affect cell viability; a more flexible and cumulative unit was chosen: energy deposition in energy per unit area. Energy deposition is composed of duration, intensity and duty cycle; many combinations can produce the same energy per unit area. It was the simplest method to find the range of ultrasound parameters which cells can withstand.

$$Energy\ Deposition = \ Time * \ Intensity * \ Duty\ cycle = s * \frac{J/s}{cm^2} * \% = \frac{J}{cm^2}$$

The cell line that was chosen for this test was the adherent MCF-7. They are less susceptible to the damaging effects of sonoporation than suspended MCF-7 or the KG-1 cells. As mentioned in previous chapter, $1.5x10^5$ cells in 350uL of nutrient-rich medium were seeded the day before the experiment. The cells were treated with varying ultrasound energy depositions; 400, 800, 1200, 1800, 2000, 6000 mJ/cm^2. The ultrasound was generated by the Excel UltraMax commercial machine with a 1MHz transducer. The ultrasound settings for each energy depostion are listed in Table 4.1. In this experiment, no sonoporation markers were used; MTT assay was used to measure the cell viability of each sample 48 hours later.

Energy	Ultrasound Specification		
	Duration	Duty Cycle	Intensity
400 mJ/cm^2	20s	20%	0.1W/cm^2
800 mJ/cm^2	40s	20%	0.1W/cm^2
1200 mJ/cm^2	60s	20%	0.1W/cm^2
1800 mJ/cm^2	30s	20%	0.3W/cm^2
2000 mJ/cm^2	20s	20%	0.5W/cm^2
6000 mJ/cm^2	60s	20%	0.5W/cm^2

Table 4.1 - The Excel UltraMax ultrasound machine's setting for each energy deposition in Energy and Definity Concentration Test

Concurrently, microbubble concentration was also tested, as sonoporation facilitates microbubbles. Since more inertial cavitations causes more damage to cells, it is also important to investigate the appropriate concentration of Definity™, the primary microbubbles used in this thesis. The amount of Definity™ tested was 0, 40, 80, 100 and 120uL per dish. The six energy

deposition conditions in Table 4.1 were performed for each concentration of Definity™, hence there will be five sets of data. Samples with Definity™ but not given ultrasound treatment were set as negative controls and as an zero for the MTT assay. The cell death in the controls will not be caused by sonoporation, but by natural causes, hence, they would simulate growth of normal cells without disturbance. All other MTT assay readings will be compared with the controls.

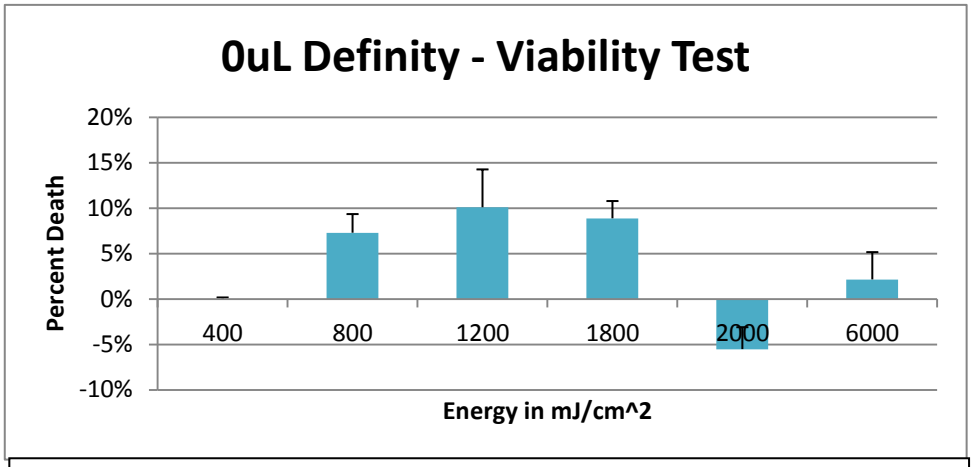

Figure 4.1 - MTT/Cell viability results for Controls in Energy and Definity concentration test

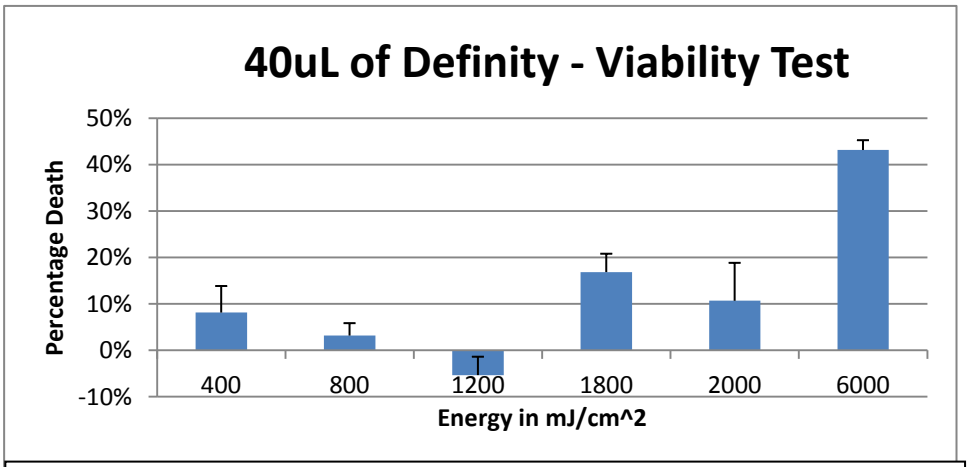

Figure 4.2 - MTT/Cell viability results on 40uL of Definity in Energy and Definity concentration test

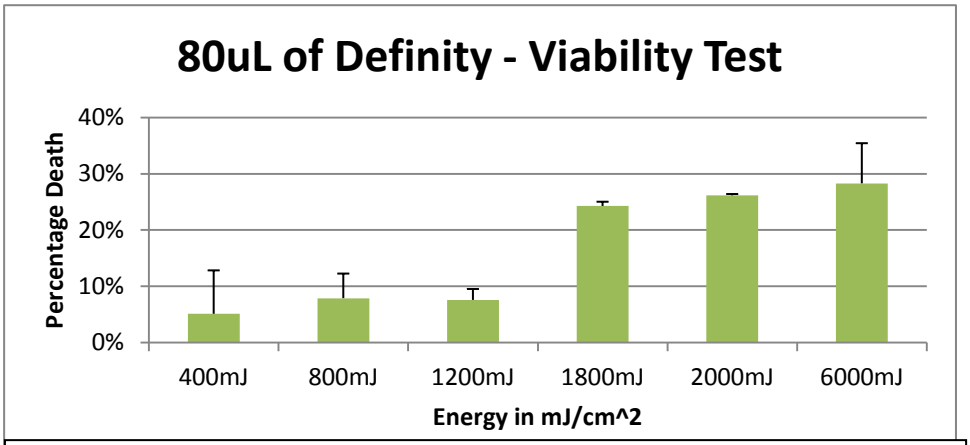

Figure 4.3 - MTT/Cell viability results on 80uL of Definity in Energy and Definity concentration test

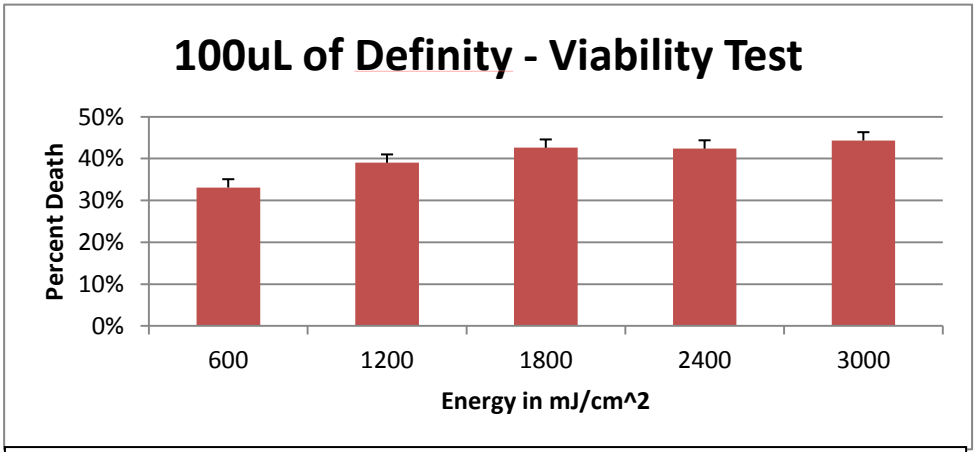

Figure 4.4 - MTT/Cell viability results on 100uL of Definity in Energy and Definity concentration test

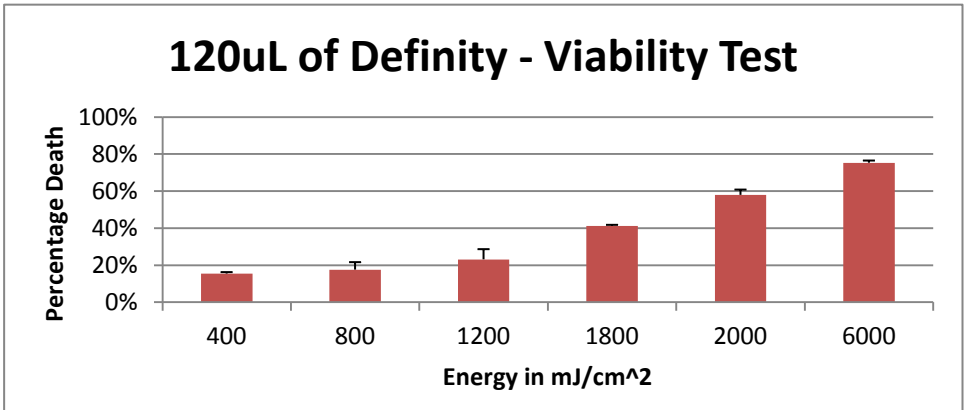

Figure 4.5 - MTT/Cell viability results on 120uL of Definity in Energy and Definity concentration test

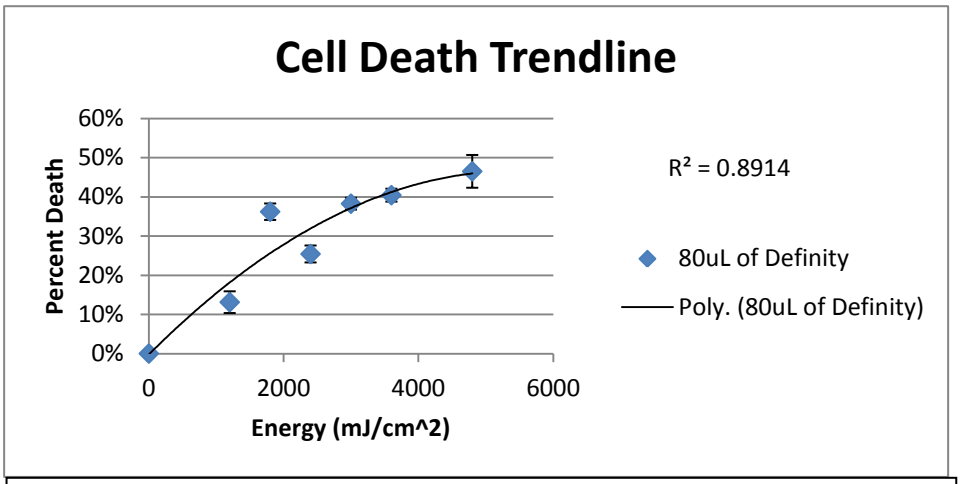

Figure 4.6 - Trend line of cell death for 80uL of Definity per dish under various ultrasound deposition energies

The result conformed to the two expected trends. The higher the concentration of Definity in each dish, the higher the rate of cell death; and the higher the ultrasound energy, the more likely the cells will not survive.

From figure 4.1 represents samples which were subjected to ultrasound without Definity™. The viability of this group did not drop below 85%.. This group of studies suggests that Definity™ is an important factor in causing the reported trend seen in sonoporation experiments and that Definity™ is the determinant in causing cell death: if Definity™ is not present, there will be minimal cell death. Since cell death is a side effect of sonoporation, without significant cell death in groups shown in figure 4.1, it may also suggest that without Definity™ there will be minimal sonoporation activity and transfection.

The data on figure 4.2 represent the amount of cell death caused by 40uL of Definity™ in each dish with 700uL of medium. The samples have cell death always below 25% with the exception of the strongest ultrasound energy deposition, with cell viability being 40%. This excessive cell death suggests that 6000mJ/cm^2 may not be suitable for ultrasound energy deposition conditions. Furthermore, in figure 4.2, 4.3 and 4.5, there was an increase in cell death

between samples of 1200 and 1800 mJ/cm^2. The cell viability was almost the same between 400, 800 and 1200 mJ/cm^2. This may have been caused by the change of ultrasound intensity for the two ultrasound energy deposition conditions. 400, 800 and 1200 mJ/cm^2 was generated with 0.1W/cm^2, while 1800 mJ/cm^2 were generated with 0.3W/cm^2. However, this trend expected is not apparent in the data presented in Figure 4.4. This may suggest that the threshold intensity for Definity™ microbubbles to inertial cavitate should be between 0.2 and 0.3W/cm^2 at 1MHz ultrasound generated by the Excel UltraMax ultrasound machine.

Other samples from 100uL and 120uL of Definity™ data sets also suggest that 100uL and above may not be a suitable concentration of Definity™ for further testing due to the high rate of cell death and the extensive increase in cell death with minute changes in energy deposition. Therefore, 80uL of Definity™ was determined to be the limit which could be added to each dish. To further study the relationship between cell death and energy deposition, Figure 4.6 was made from the data obtained from the 80uL Definity™ per dish set. The data suggests that cell death will reach a plateau as energy deposition increases. This supports the hypothesis that cell viability is greatly dependent on concentration of Definity™ rather than energy deposition, as long as ultrasound intensity passes the cavitation threshold.

4.1.2 - Volume of Definity™ Per Dish

As it was established in the previous experiment, Definity™ is the more dominant factor in determining cell viability than ultrasound energy deposition. The volume of Definity™ is related to the amount of microbubbles, $1.2x10^{10}$ bubbles/mL [91]. The following experiments are based on the ratio of cells to microbubbles.

To explore the ratio between cells and microbubbles, two sets of experiments were performed. The amount of medium per dish was held constant for both sets, while one set had various cell numbers with the amount of Definity™ constant. $8x10^4$, $1x10^5$ or $1.5x10^5$ cells in 700uL of medium were sonoporated with 40uL of Definity™ per dish. Each condition had five samples treated with different ultrasound energy deposition conditions: 0, 600, 1200, 1800, and 2400 mJ/cm^2. The ultrasound was administered by the Excel UltraMax ultrasound commercial box with 1MHz trandsucer as in previous experiments. The intensity was set at 0.3W/cm^2 and 20% duty cycle. The details of the ultrasound specifications are listed on Table 4.2.

The second set of experiments held the cell popultation constant, while amount of Definity™ per dish was altered. 40, 80 or 120uL of Definity™ was added to each dish with $1.5x10^5$ cell. The cells were treated in ultrasound with four different conditions of energy depositions: 0, 600,1200 and 1800 mJ/cm^2. The ultrasound was also administered by the Excel UltraMax ultrasound commercial box at 1MHz with intensity set at 0.3W/cm^2 and duty cycle at 20%.

Energy	Ultrasound Specification		
	Duration	Duty Cycle	Intensity
600 mJ/cm^2	10s	20%	0.3W/cm^2
1200 mJ/cm^2	20s	20%	0.3W/cm^2
1800 mJ/cm^2	30s	20%	0.3W/cm^2
2400 mJ/cm^2	40s	20%	0.3W/cm^2

Table 4.2 - The Excel UltraMax ultrasound machine's setting for each energy deposition in Concentration of Definity

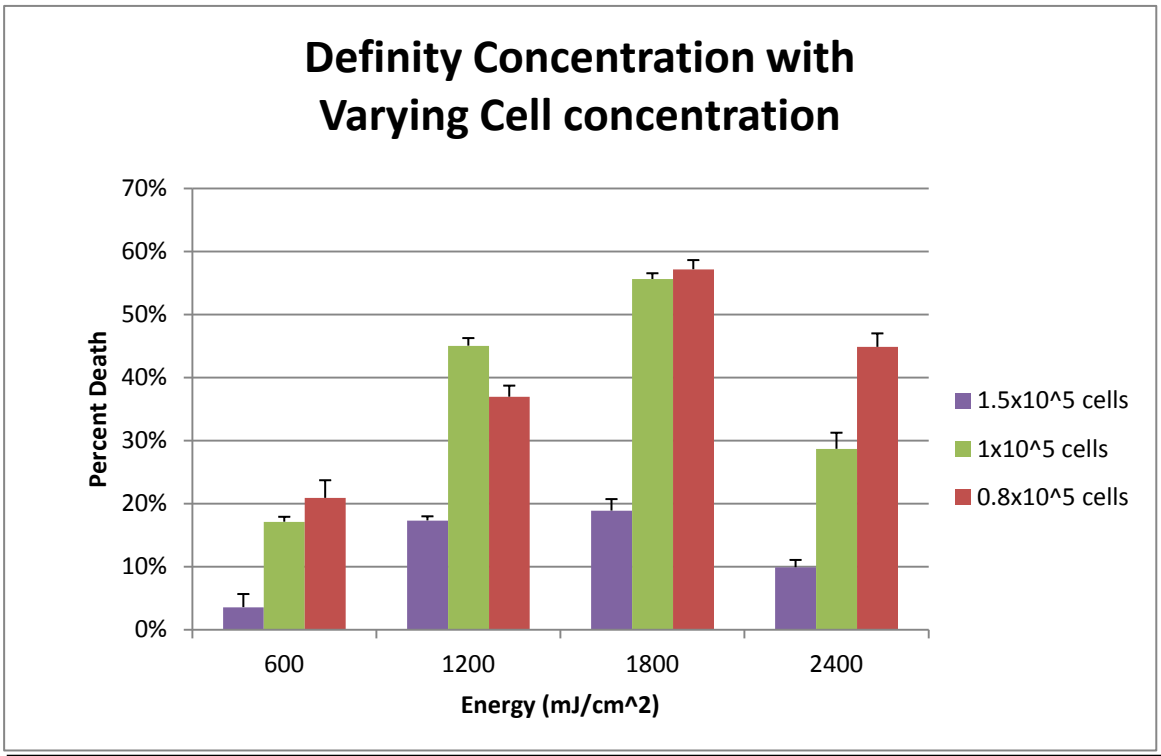

Figure 4.7 - Different number of cells seeded with 40uL of Definity in various ultrasound energy depositions.

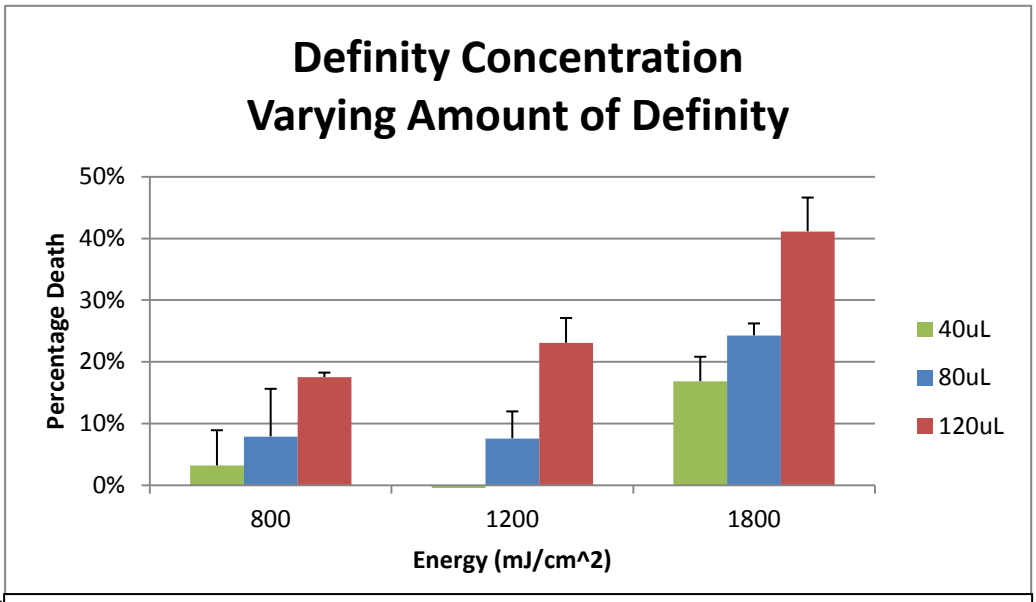

Figure 4.8 - Different uL of Definity with $1.5x10^5$ cells per dish, with various ultrasound energy depositions.

The results in figure 4.7 show the dramatic difference in cell viability as a function of cell numbers. This difference in cell viability between $1.5x10^5$ to the $1x10^5$ and $0.8x10^5$ is substantial. The samples with $1.5x10^5$ cells in the beginning are less likely to be killed by collateral damage done by the sonoporation. A decrease in cell number from $1.5x10^5$ to $1x10^5$ cells per dish can cause as much as 30% more cell death. However, when analyzing Figure 4.8 where the cell number was held constant, the difference between 40 and 80uL of Definity™ in solution, does not bring about such a drastic increase in cell death as seen in figure 4.7.

From these two tests; it was hypothesized that changing the number of cells seeded into each dish will have a large determinant effect on cell viability. Therefore, it would be wise to vary cell number in the search for an ideal range of parameters where transfection using sonoporation is successful and with minimal cell deaths. After a crude range of parameter

settings was determined for one cell line, then adjusting the amount of Definity per dish can further optimize sonoporation .

These results also gave some insight of the characteristics of sonoporation with Definity. There are two ways to change the ratio of microbubbles to cells as previously mentioned. However, the two are not equivalent when the volume of medium is held constant. Lowering the cell to microbubbles ratio via lowering cell numbers implies each cell will, on average, occupy more volume. The increase in average occupying volume will lead to an increase in average distance between cells and microbubbles. Lowering cell to microbubbles ratio can also be achieved via increasing amount of bubbles added. This leads to a higher concentration of microbubbles and a decrease in the average distance between microbubbles and the cells. One would expect a decrease in average distances would cause more cell deaths as the shockwaves and radicals generated by microbubbles cavitation are more detrimental at smaller distances. The two sets of experiments show that decreasing the average distance (figure 4.8) is less detrimental than increasing the average distance (figure 4.7). This implies that the assumption about the difference in average distance is not valid. A possibility for such a phenomenon is that the microbubbles are not evenly spaced within the medium, but are rather attracted and attached to the cell membrane. This suggests that waiting 15 minutes before sonication gives the Definity™ microbubbles an opportunity to find attach themselves to the adherent MCF-7 cells, increasing the efficacy of the microbubbles.

4.1.3 - GFP Concentration Test

The preliminary selection for the amount of ultrasound energy deposition, volume of Definity™ per dish and the number of cells seeded in each dish were determined based on the previous experiments. In this test, these parameters were tested for their ability to transfect cells. Ultrasound was applied to adherent MCF-7 cells in medium containing Definity™ and GFP. GFP plasmid was used as a transfection plasmid and transfection marker; GFP plasmid will have to enter the nucleus where it will be transcribed into mRNA, and the mRNA will then have to be translated into a protein product by ribosomes in the cytoplasm of the cell. The protein product will emit green photons in 500nm range when irradiated by a blue light at 480nm. This green emission was recorded and analyzed by the use of flow cytometry. This process will demonstrate the ability to transfect cells within the previously established parameters. An example of a control (or negative) and a positive flow cytometry results are shown in Figure 4.9.

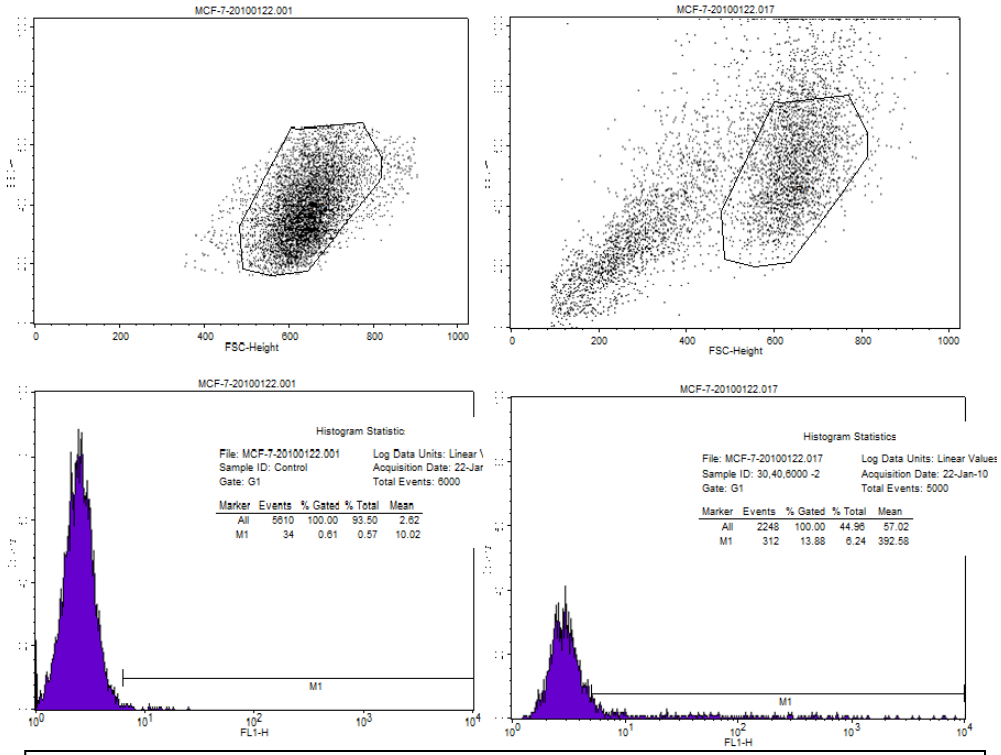

Figure 4.9 - An example of flow cytometry results from a typical control (left) and a positive result with 13% transfection rate (right).

In this experiment, three conditions were selected from the established range of parameters. 40uL of Definity™ with 1800 mJ/cm^2, produced by the 1MHz transducer of the Excel UltraMax commercial ultrasound box at 0.3W/cm^2 and 20% duty cycle for 30s, was one of the settings which will most likely lead to transfection while preserving viability. Since the setting had never been tested for transfection efficiency, a higher ultrasound intensity with more Definity™ were included to ensure ultrasound mediated transfection was possible. The other two conditions were 140uL of Definity™ with 6000 mJ/cm^2 of ultrasound, and 40uL of Definity™ with 6000 mJ/cm^2 of ultrasound; the 6000 mJ/cm^2 were produced by the 1MHz transducer of the Excel UltraMax commercial ultrasound box at 0.5W/cm^2 and 20% duty cycle for 60s. These two settings were expected to have high cell death rates. Another positive control used was PEI (polyethylenimine); this was used to demonstrate the ability of GFP expression by the cell line.

The experiment was repeated four times. The three readings were accounted for and averaged. Results are displayed on Figure 4.10.

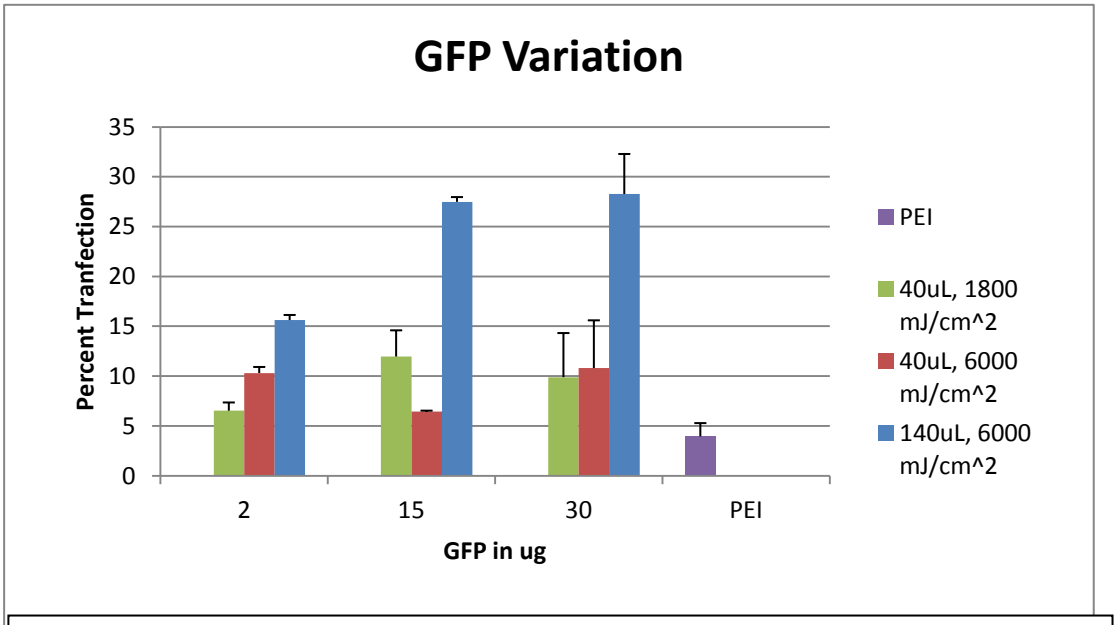

Figure 4.10 - Different amount of GFP with 1.5x10^5 MCF-7 cells per dish in various sonoporation parameters from GFP Concentration Test

From Figure 4.10, the results suggest that transfection can occur in all three sonoporation parameters. Furthermore, these sonoporation parameters are transfecting better than the positive controls, the chemical tranfecting agent, PEI.

Figure 4.10 also enforces the previously belief that Definity™ concentration is a dominant determinant for cell viability and transfection. Samples were significantly more successfully transfected when they were sonoporated with 140uL of Definity™ instead of 40 uL($p<0.05$). However, the cell viability of cells sonoporated using 140uL of Definity™ was expected to be unacceptably high, and will not be used as a standard. The figure also shows that the transfection rate in samples treated with 40uL and 1800mJ/cm^2 are comparable to those treated with 40uL and 6000mJ/cm^2. Therefore, the result suggests that the excessive energy deposited at a higher intensity and longer duration of ultrasound did not increase the transfection in adherent MCF-7 cells.

From the data, there are little evidences showing the difference between samples sonoporated with 15ug and 30ug of GFP plasmid. There are some differences in transfection between samples transfected with 2ug and 15ug, but the trend is unclear. It can be generalized that GFP concentration's effects on transfection plateaus beyond 15ug. Since GFP is relative expensive and time consuming to produce, therefore, 15ug of GFP will be used in the future experiments.

From this experiment, a sonoporation experiment standard for this thesis was established: adherent MCF-7 cells can be transfection with about 12% success rate when sonoporated at 0.3W/cm^2 and 20% duty cycle for 30s with 40uL of Definity™ and 15ug of GFP plasmid, with a viability of at least 80%.

4.1.4 - Exploration of Ultrasound Parameters

In previous experiments, ultrasound parameters were lumped together as one parameter, energy deposition. However, it was uncertain how each of these ultrasound parameters affects sonoporation and cell viability. As previously mentioned, there are several parameters that could be changed in producing the same energy deposition; they are intensity, frequency, duration, duty cycle and pulse repetition. Frequency and pulse repetition are dependent on machine's specification, therefore, they are difficult to assess.

This experiment was planned, performed and analyzed with the assistance from Mr. Michael Choi, a graduate student at the Electrical Engineering Department at University of Alberta. The two energy deposition used to explore the effects of ultrasound intensities, duty cycle and duration were 1800 and 6000 mJ/cm^2. Since there are three factors which contribute to the amount of energy deposited into the samples, it is only possible to hold one variable constant when holding energy deposition constant; the other two factors will be varied.

There will be four sets of data; duty cycle was held constant for two sets each at different amount of energy deposition, and intensity was held constant for two sets each at different amount of energy deposition. The details on the parameters used to generate 1800 and 6000mJ/cm^2 with the 1MHz transducer from the Exel UltraMax commercial ultrasound box can be found on Table 4.3. $1.5x10^5$ MCF-7 cells were seeded in to the centre of the 35mm dish the day before experiment, and 40uL of Definity™ was added to each sample 15 minutes before the experiment to facilitate sonoporation.

6000mJ/cm^2	W/cm^2	Duration (Sec)	% Duty Cycle
With constant 20% Duty Cycle	0.1	300	20%
	0.3	100	
	0.5	60	
	0.7	43	
	0.9	33	
	1.5	20	
	1.7	18	
	2	15	
With Constant Intensity 0.5W/cm^2	0.5	120	10%
		60	20%
		40	30%
		24	50%
		12	100%
1800mJ/cm^2	W/cm^2	Duration (Sec)	% Duty Cycle
With constant 20% Duty Cycle	0.1	90	20%
	0.3	30	
	0.5	18	
	0.9	10	
With Constant Intensity 0.5W/cm^2	0.3	60	10%
		30	20%
		20	30%
		12	50%
		6	100%

Table 4.3 - Detail on how ultrasound at 6000 and 1800 mJ/cm^2 were generated in Exploration of Ultrasound Parameter

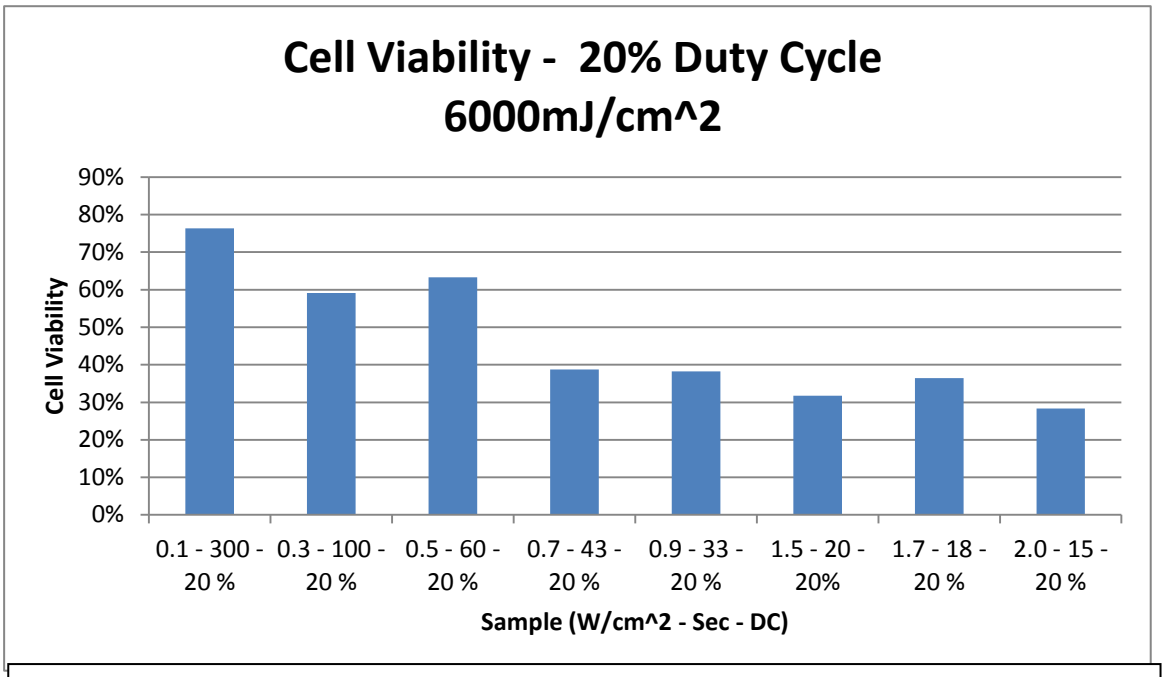

Figure 4.11 - Viability of Cells after treated with 6000mJ/cm^2 of ultrasound deposited with different parameters while holding Duty Cycle constant with 40uL of Definity in Exploration of Ultrasound Parameters

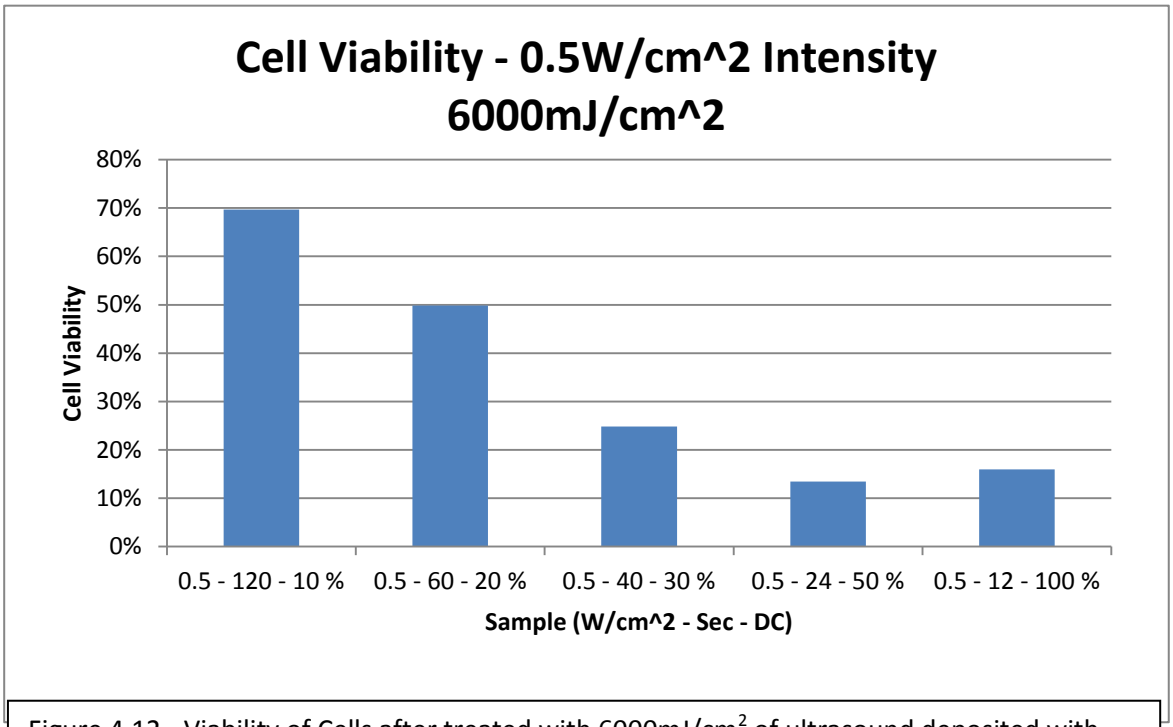

Figure 4.12 - Viability of Cells after treated with 6000mJ/cm^2 of ultrasound deposited with different parameters while holding intensity constant with 40uL of Definity in Exploration of Ultrasound Parameters

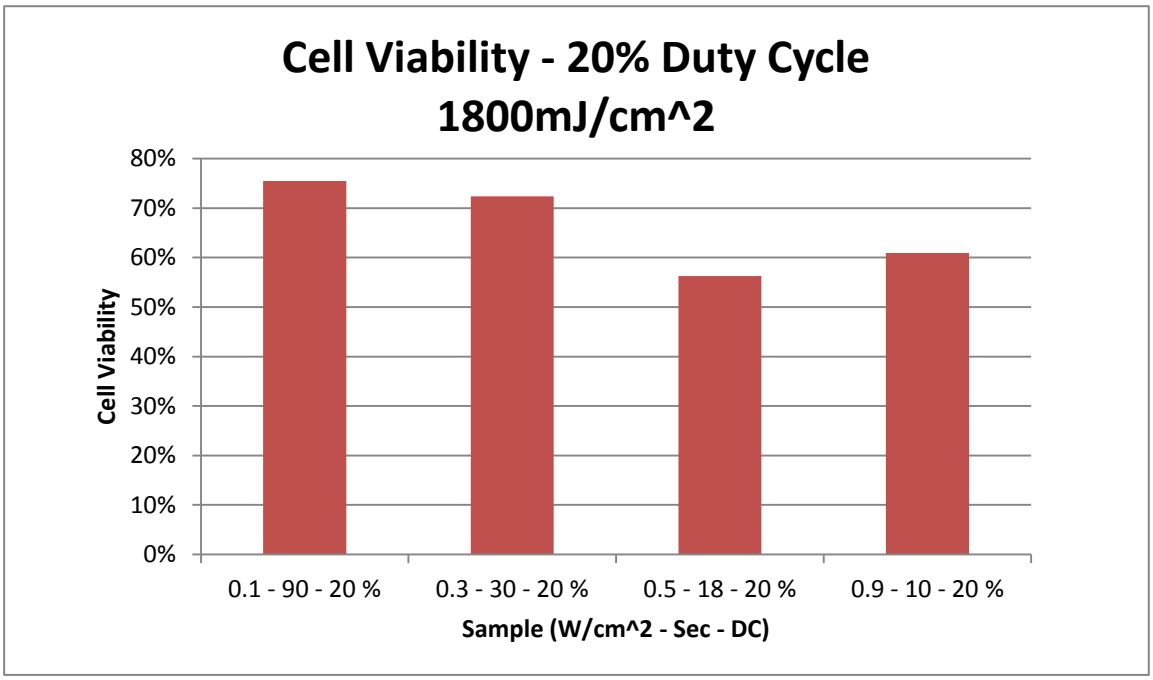

Figure 4.13 - Viability of Cells after treated with 1800mJ/cm^2 of ultrasound deposited with different parameters while holding Duty Cycle constant with 40uL of Definity in Exploration of Ultrasound Parameters

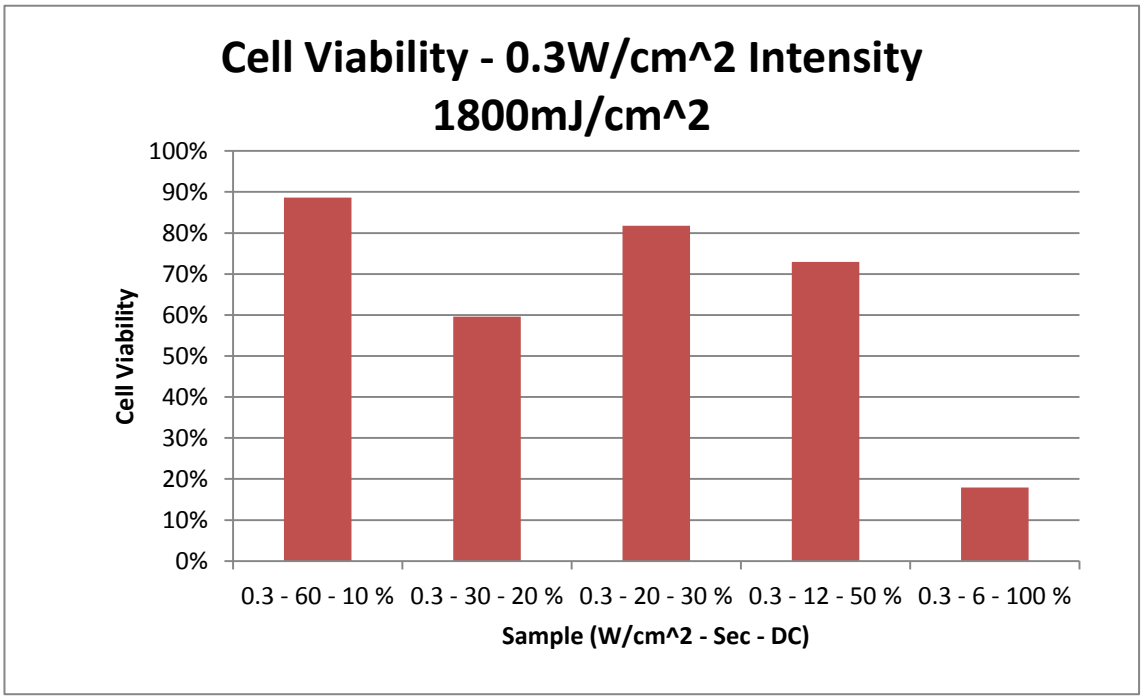

Figure 4.14 - Viability of Cells after treated with 1800mJ/cm^2 of ultrasound deposited with different parameters while holding intensity constant with 40uL of Definity in Exploration of Ultrasound Parameters

From the results on Figure 4.12, it shows that as duty cycle increased, more cell death resulted. This is not as evident for lower energy deposition at 1800mJ/cm^2. However, Figure 4.14 supports the hypothesis that continuous ultrasound is not suitable for sonoporation as it leads to high death rate. Furthermore, Figure 4.12, demonstrates that cell viability drop off quickly for 50% duty cycle. It suggests that 20% duty cycle would be the highest duty cycle the ultrasound should be set at.

Secondly, from Figure 4.11 and 4.13, the data suggest higher intensity causes higher death rate. This confirms the trend shown in previous experiments. The results on Figure 4.11 also suggest that intensity should not be higher than 0.5W/cm^2 as the cell viability drop below 40% viability at 0.7W/cm^2. Furthermore, the results also imply that intensity has a stronger impact on cell viability than duration. Therefore it is safer for cells to go through a longer period of ultrasound than at a higher intensity to achieve the same ultrasound energy deposition.

The results from this experiment confirm the theories and trend lines previously assumed. A lower intensity generated by duty cycle less than 20% with longer sonication period will preserve viability better.

4.2 - Transition into Suspension Cells

4.2.1 - Suspension MCF-7

The goal of the thesis is to improve ultrasound-mediated transfection on difficult to transfect cell lines such as KG-1, therefore suspending the easy to transfect cell line would be the first step in finding out how cells in suspension react to sonoporation. The suspension of adherent cells is not a new idea. In various literatures, adherent cells were suspended before they were sonoporated [94, 95].

The suspended MCF-7 was treated with the same experimental conditions as adherent cells from previous experiment. Instead of seeding $1.5x10^5$ cells the day before the experiment, cells were cultured and $1.5x10^5$ cells were placed in 350uL of basal medium the day of experiment. 40uL of Definity™ was added to the 350uL of medium. The medium was transferred to the center of the dishes mounted on the 1MHz transducer of the Excel UltraMax commercial ultrasound machine. The samples were sonoporated with 600, 1200, 1800, 2400 and $3000mJ/cm^2$ of energy deposition using intensity of $0.3W/cm^2$; the details of the parameters used for each energy deposition are recorded on Table 4.4.

Cell viability was tested with MTT assay the next day and Optical Density (OD) was also recorded. The results were compared along side with samples from pervious experiment from section 4.1.2 on Figure 4.15. Both of these samples were sonoporated using the exact same conditions: $1.5x10^5$, 40uL of Definity™, subject to $0.3W/cm^2$, and 20% duty cycle ultrasound. The only difference between the two samples was the state the MCF-7 cells were in during sonoporation.

Energy	Ultrasound Specification		
	Duration	Duty Cycle	Intensity
600 mJ/cm^2	10s	20%	0.3W/cm^2
1200 mJ/cm^2	20s	20%	0.3W/cm^2
1800 mJ/cm^2	30s	20%	0.3W/cm^2
2400 mJ/cm^2	40s	20%	0.3W/cm^2
3000 mJ/cm^2	50s	20%	0.3W/cm^2

Table 4.4 - The Excel UltraMax ultrasound machine's setting for each energy deposition in Suspension MCF-7

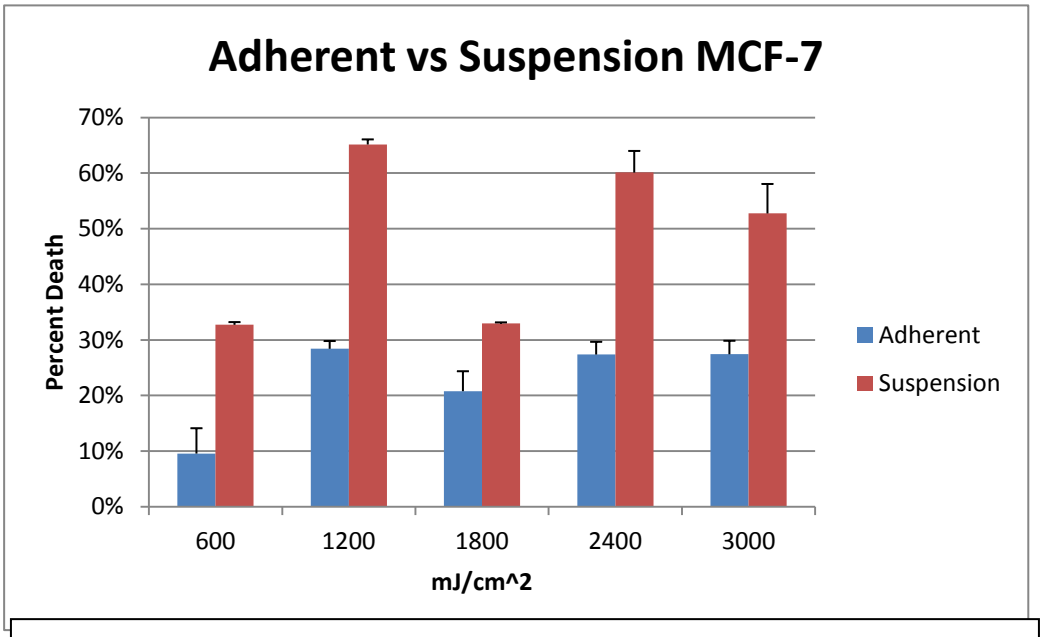

Figure 4.15 - The amount of cell death for adhered and suspended MCF-7 samples 48 hr after sonoporation at various ultrasound energy depositions with 40uL of Definity when 1.5x10^{5}cells were seeded.

The results suggest that when cells are in suspension, they will be more likely to die after sonoporation. They were believed to be damaged more severely by cavitation because there are more surface areas of the cell that are surrounded by microbubbles. This huge discrepancy on cell viability between adhesion and suspended MCF-7 cells under the same

sonoporation conditions, suggest that suspended MCF-7 should be treated as a new cell line.

After trial and error, an optimal range for suspended MCF-7 was found. The ultrasound parameters were kept the same, but the concentration of Definity™ and cell numbers were altered. The number of cells was increased from $1.5x10^5$ to $3x10^5$ and the amount of Definity™ was dropped to 20uL per dish.

4.2.2 - Ultrasound Machines

4.2.2.1 - Suspended MCF-7

In order to test for the effects of ultrasound frequency and pulse repetition, it is necessary to use two different ultrasound systems. The Excel UltraMax commercial ultrasound machine was compared to the homebrew ultrasound box based on SonaCell. SonaCell is an ultrasound machine that was developed and built by Dr. Jie Chen's lab and was sold under the company IntelligentNano. The SonaCell uses different frequency and have a different pulse repetition rate than the Excel UltraMax. The SonaCell produces 1.5MHz ultrasound at 1Hz repetition rate; details were presented in Chapter 3 of this thesis.

The suspended MCF-7 cells were used to explore the difference between the two ultrasound machines. The concentration of Definity used was 20uL, with $3x10^5$ cells per in 350uL of basal medium. Both of the ultrasound machines were tested with energy deposition of 600, 900, 1200 and 1800 mJ/cm^2. However, the ultrasound parameters which generate the energy deposition for both machines were not exactly the same; the details on the parameters used can be found on Table 4.5.

Transfection with GFP plasmid was tested in both of these ultrasound machines. 15ug of GFP was added to the samples before transfection. The results were analyzed 48 hours later by flow cyctometry. The percentages of fluorescent cells were compared against each other on Figure 4.16. MTT assay was also done on these samples, and the results between the two ultrasound boxes were compared on Figure 4.17.

Energy	Excel Ultra Max			SonaCell		
	Duration	Duty Cycle	Intensity	Duration	Duty Cycle	Intensity
600 mJ/cm^2	30s	10%	0.2W/cm^2	30s	10%	0.2W/cm^2
900 mJ/cm^2	30s	10%	0.3W/cm^2	45s	10%	0.2W/cm^2
1200 mJ/cm^2	30s	20%	0.2W/cm^2	30s	20%	0.2W/cm^2
1800 mJ/cm^2	30s	20%	0.3W/cm^2	45s	20%	0.2W/cm^2

Table 4.5 - The Excel UltraMax ultrasound machine's and SonaCell ultrasound machines setting for each energy deposition.

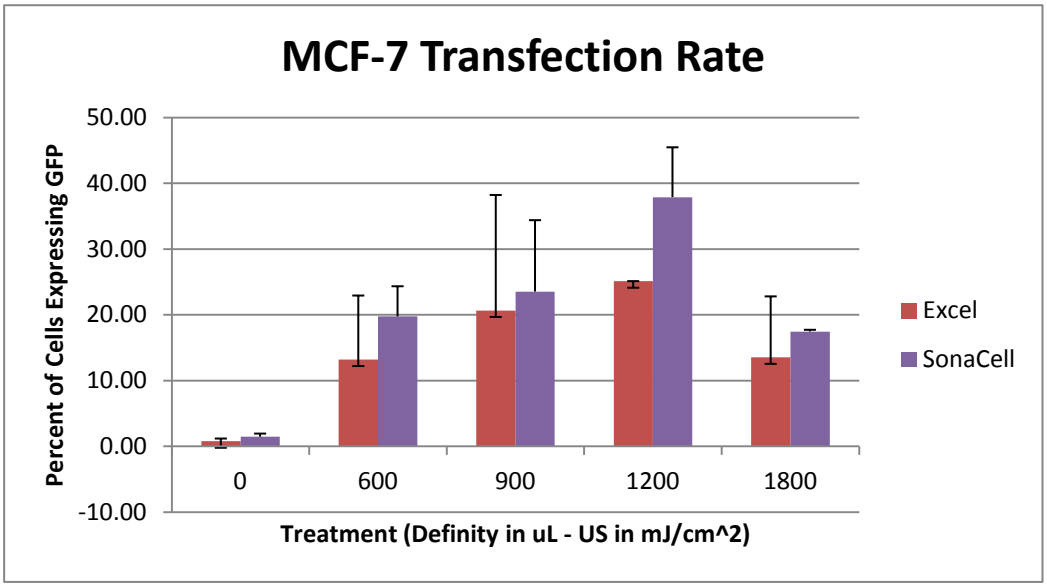

Figure 4.16 - Transfection rate of suspended MCF-7 cells sonoporated under two different ultrasound platforms.

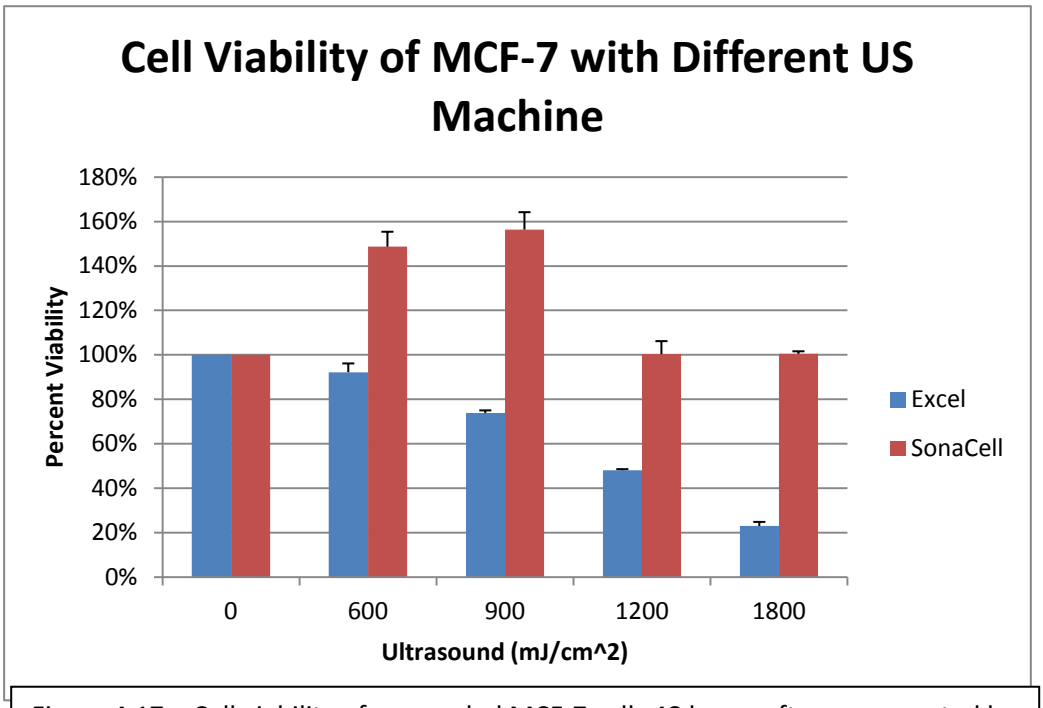

Figure 4.17 – Cell viability of suspended MCF-7 cells 48 hours after sonoporated by two different ultrasound platforms.

From Figure 4.17, it is obvious that suspended MCF-7 responded differently to the two ultrasound machine. The cell viability of the SonaCell box is much higher than that of the Excel UltraMax commercial ultrasound box. More surprisingly, cells that were sonoporated by the SonaCell have uncompromised or better viability than the controls. This increase in cell viability by SonaCell was not expected. This phenomenon might have been due to the enhancement of cell proliferation by ultrasound. The SonaCell was originally developed for stem cell proliferation. The ultrasound generated by the SonaCell box is capable of increasing the stem cells number and help maintain their overall health. However, the experiment protocol for stem cell proliferation is not the same as this experiment. For stem cell proliferation, the SonaCell produces ultrasound waves that massage the cells for 10 minutes each day, without the use of

microbubbles. This was a surprising finding to observe the proliferation properties of the SonaCell under different ultrasound application parameters and circumstance.

Even though the SonaCell ultrasound box improves viability of ultrasound, the sonoporation dilemma was still unsolved. The Excel UltraMax commercial ultrasound machine, with lower cell viability, outperformed the SonaCell. This again confirms the hypothesis that sonoporation is a form of damage, and the more intense the sonoporation the more likely the cells will not survive. However, figure 4.16 may also suggest that the optimal level of transfection does not necessary imply the highest energy deposition. The highest transfection rate for Excel UltraMax ultrasound machine occurs at 900mJ/cm^2, while the best transfection occurs at 1200mJ/cm^2 for SonaCell. Forbes confirmed this hypothesis in her paper in 2011[96]. Forbes showed that sonoporation increases as intensity of ultrasound increases, but drastically decreases after reaching its cavitation threshold. Forbes believes that microstreaming is the dominant mechanism which leads to the increase in cell membrane permeability; with all the bubbles bursted through inertial cavitation, there are no bubbles left to perform stable cavitation or microstreaming. Forbes' paper helps explain the results from Figure 4.16 of why higher energy depositions can lead to more cavitation and more cell death, but does not lead to the optimal transfection rate; microstremeaming caused by stable oscillations influence sonoporation as well.

4.2.2.2 - KG-1 Cells

Different cell lines have different responses to ultrasound treatment; this was true even for adherent and suspension form of MCF-7 cell. Therefore, it was essential to empericially test the KG-1 with different ultrasound systems to determine which system is suitable for KG-1 trnasfection. In this experiment, $7.5x10^5$ KG-1 cells were suspended in 350uL of medium with 20uL of Definity™ and 15ug of GFP plasmid. The samples were treated with three frequencies: 1.5MHz from SonaCell, 1MHz and 3MHz from Excel UltraMax commerical ultrasound box. The 3MHz ultrasound comes from the same transducer as the 1MHz ultrasound and both have repetition rate of 100Hz. The settings for each energy deposition conditions with each ultrasound system were the same as previous experiment, which is listed in Table 4.5; the intensity, duration and duty cycle settings for 3MHz at each energy deposition were the same as the scheme for 1MHz. The KG-1 samples were analyzed 72 hours later. The transfection results and the cell viability are compared in Figure 4.18 and 4.19 respectively.

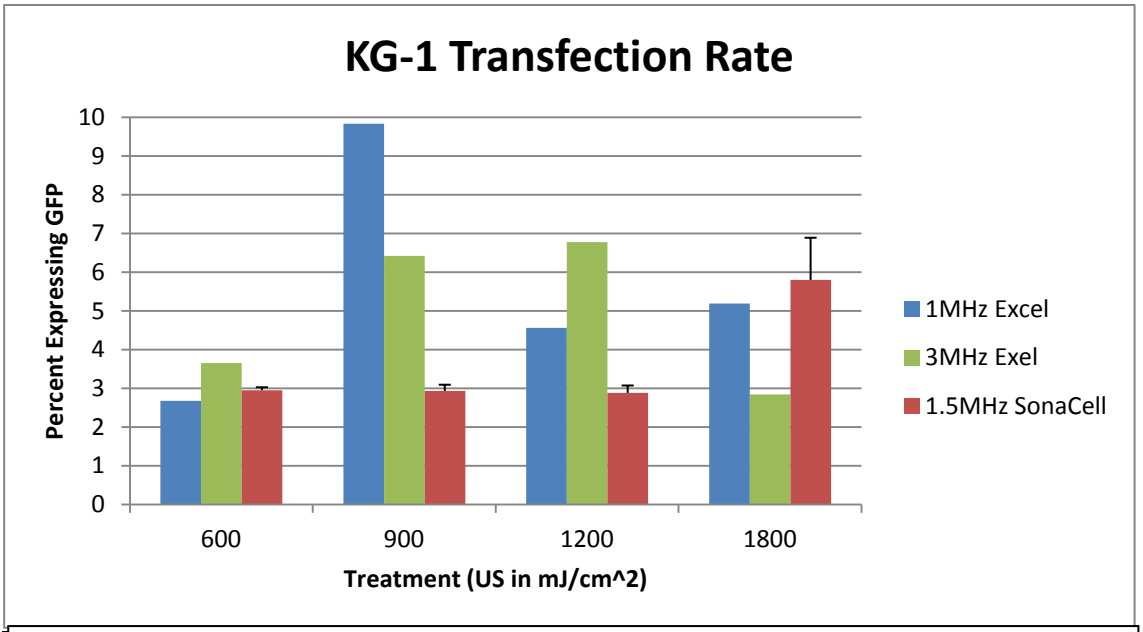

Figure 4.18- Transfection rate of KG-1 cells sonporated with three different frequencies from two different ultrasound platforms. $7.5x10^5$ cells were seeded into each sample with 20uL of Definity and 15ug of GFP plasmid.

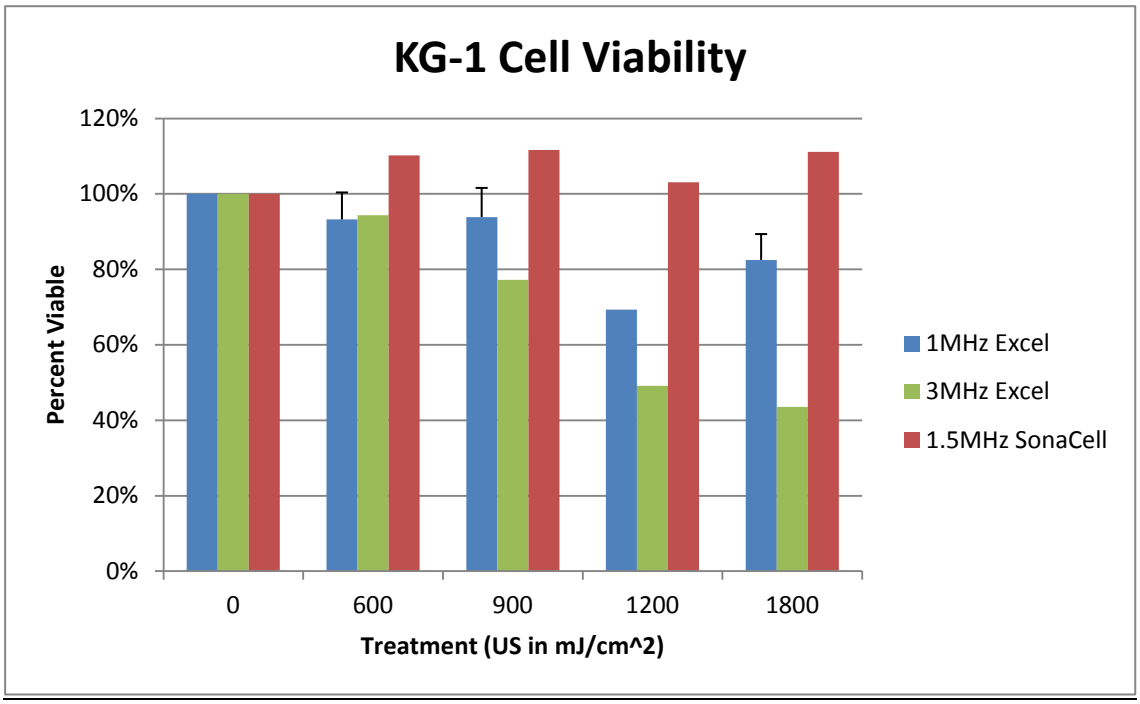

Figure 4.19 - Viability of KG-1 cells sonoporated under two different ultrasound platform and three different frequencies. $7.5x10^5$ cells were seeded into each sample with 20uL of Definity.

The results on figure 4.18 confirm the difficulty in transfecting KG-1 cells. The conditions did not bring about any significant transfection. None of the samples have transfection rate higher than 10%. The highest transfection comes from the sample administered with 1MHz ultrasound at 900mJ/cm^2. The highest tranfection rate of approximately 6% for 3MHz ultrasound occurred at 1200mJ/cm^2. In general, the 1.5MHz ultrasound generated by SonaCell has the weakest transfection; approximately 5% at 1800mJ/cm^2. The results again assure that there is an optimal energy for sonoporation; higher energy does not necessary lead to a stronger transfection. The results indicate that the optimization of sonoporation varies with frequency, which directly affects the cavitation behavior of microbubbles; again coincide with Forbes' theory.

The viability of the samples were displayed in Figure 4.19. The viability drops drastically for the two frequencies produced by the Excel Ultramax ultrasound box at 1200mJ/cm^2 which

was generated with 20% duty cycle, as compared to 900mJ/cm^2 which was generated with 10% duty cycle. This may suggest the KG-1 cells can only be sonoporated by Excel UltraMax at 10% duty cycle. 1.5MHz ultrasound generated by SonaCell does not exhibit the same trend. Conversely, it preserved viability and aided KG-1 proliferation. The SonaCell again demonstrates its ability to proliferate cells; a 30 seconds treatment will already benefit from its function as a cell proliferation ultrasound box. The transfection rate for 1.5MHz may be weak, but with the proliferation ability, a higher energy deposition or more Definity™ can be applied to the samples, which may prove to be more efficient in sonoporation. The optimal point of transfection is unknown as the transfection rate for 1.5MHz in Figure 4.18 continued to increase even at 1800mJ/cm^2. Therefore, further exploration on SonaCell is required to extend its potential.

4.2.3 - Summarization

Cell Line	MCF-7 Adhesion	MCF-7 Suspension	KG-1 Suspension
Number of cells per 350uL	$1.5x10^5$	$3x10^5$	$7.5x10^5$
Definity (microbubbles)	40uL	20uL	20uL
GFP plasmid	15ug	15ug	15ug
US Frequency	1MHz	1.5MHz	1.5MHz
Intensity	0.3W/cm^2	0.2W/cm^2	0.2W/cm^2
Energy	<3000mJ/cm^2	<1800mJ/cm^2	<1800mJ/cm^2
Max Tranfection	~35%	~20%	<10%

Table 4.6 - A summarization on the parameters used with each cell line and the highest transfection rate achieved.

The results shown on Table 4.6 are not significant accomplishments. Lipofectamine 2000 can achieve higher transfection rate with adherent and suspension MCF-7 cells with relatively high viability rate. As for the hard-to-transfect KG-1 cells, the transfection rate is higher than that of commercial chemical transfection reagents, but still relatively low. Sonoporation with Definity™ alone is inefficient in delivering genes to the hard-to-transfect cell lines, therefore, better methods or enhancements for sonoporation are needed to increase the transfection rate on KG-1 and other hard-to-transfect cells while maintain viability.

4.3 - Exploring New Possibilities

4.3.1 - Synergistic Property with Chemical Transfection Reagent

One of the techniques in biology to enhance gene delivery, is through synergy of two or more methods where both achieve the same goal, but utilizing different mechanisms. Through the combination of chemical transfection and sonoporation, it may be possible to enhance transfection rate [97]. If synergy was to happen between the two transfection methods, the synergistic results will lead to a higher transfection rate than the sum of the two methods' individual ability to transfection cells. The chemical transfection agents perform transfection exceptionally well on adherent and some suspension cell lines. A few commercial chemical transfection agents were tested on both of the suspended MCF-7 and KG-1 cells to assess the possibility for chemical transfection. $3x10^5$ MCF-7 cells were seeded in each sample; $7.5x10^5$ KG-1 cells were seeded in each sample. The protocol for chemical transfection reagents were listed in Chapter 3. Lipofectamine 2000, IBAfect and PEI were chosen; the results are shown on figure 4.20.

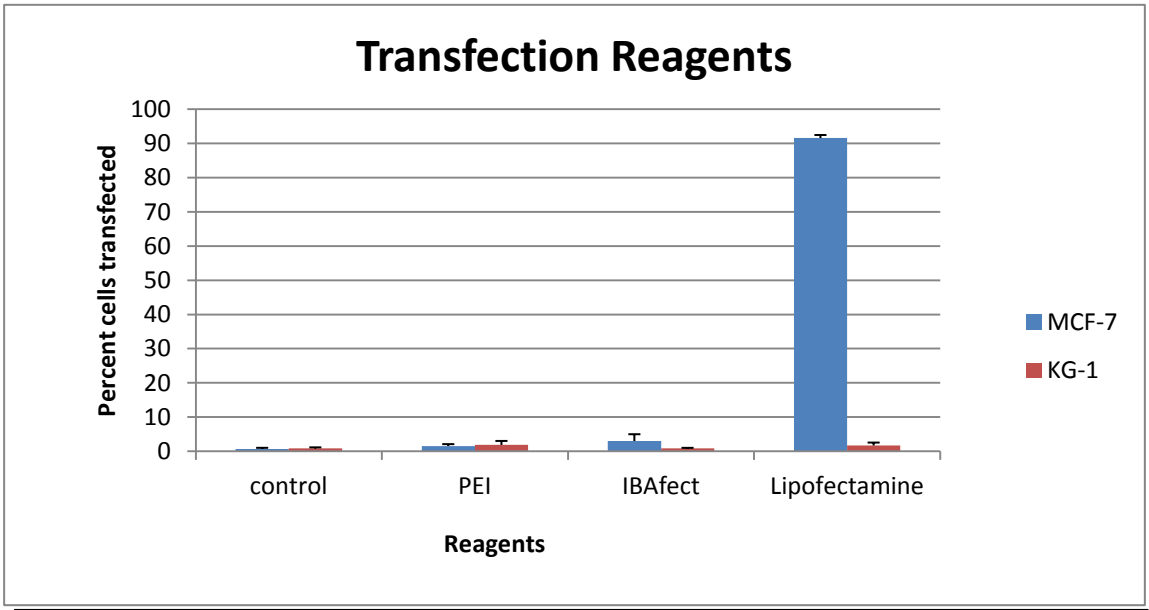

Figure 4.20 - The percent of MCF-7 or KG-1 cells expressing GFP when transfected with chemical transfection reagents: PEI (Polyethylenimine), IBAfect and Lipofectamine 2000.

The results show that none of the chemical reagents tested can transfect KG-1 cells effectively. The chemicals did not bring about higher than 5% transfection in the KG-1 cells, much weaker than sonoporation. The easy-to-transfect MCF-7 cells were effectively transfected by Lipofectamine 2000. The other chemical reagents most likely require MCF-7 cells to be adhered to the bottom of the dish in order to give higher transfection rate. Since Lipofectamine 2000 is the only efficient reagent to transfect suspended MCF-7 cells, it was used to test for synergistic properties with sonoporation.

The suspended MCF-7 cells were sonicated with 20uL of Definity™ with 1MHz ultrasound from Excel UltraMax. $3x10^5$ cells were placed in each sample. Lipofectamine 2000 was prepared as previously described in Chapter 3; Definity™ was added after the Lipofectamine samples were incubated with cells for 15 minutes. The energy deposition conditions were: 600, 900 and 1800mJ/cm^2. The ultrasound parameter settings can be referred to in Table 4.5. Flow cyctometry analysis was done 72 hours after experiment and the results are displayed in Figure 4.21. The MTT assay was also done 72 hours after the experiment and the data are and the data are displayed in Figure 4.22.

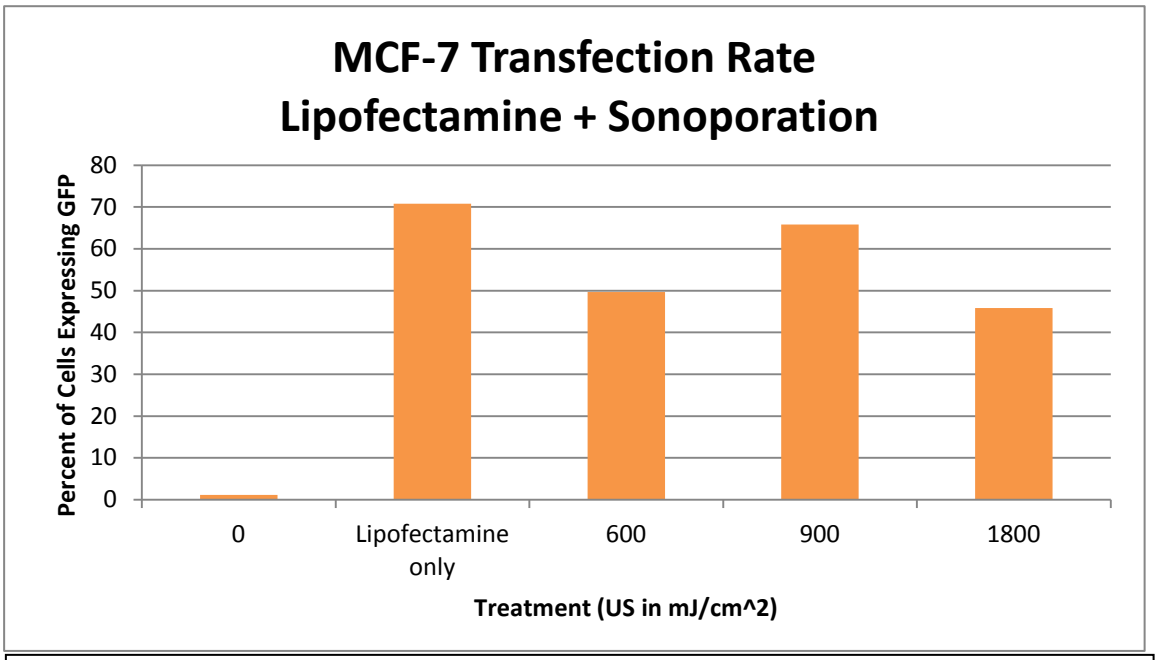

Figure 4.21 - The percent of suspended MCF-7 cells transfected 72 hours after Lipofectamine 2000 was added to cells before they were sonoporated together with Definity.

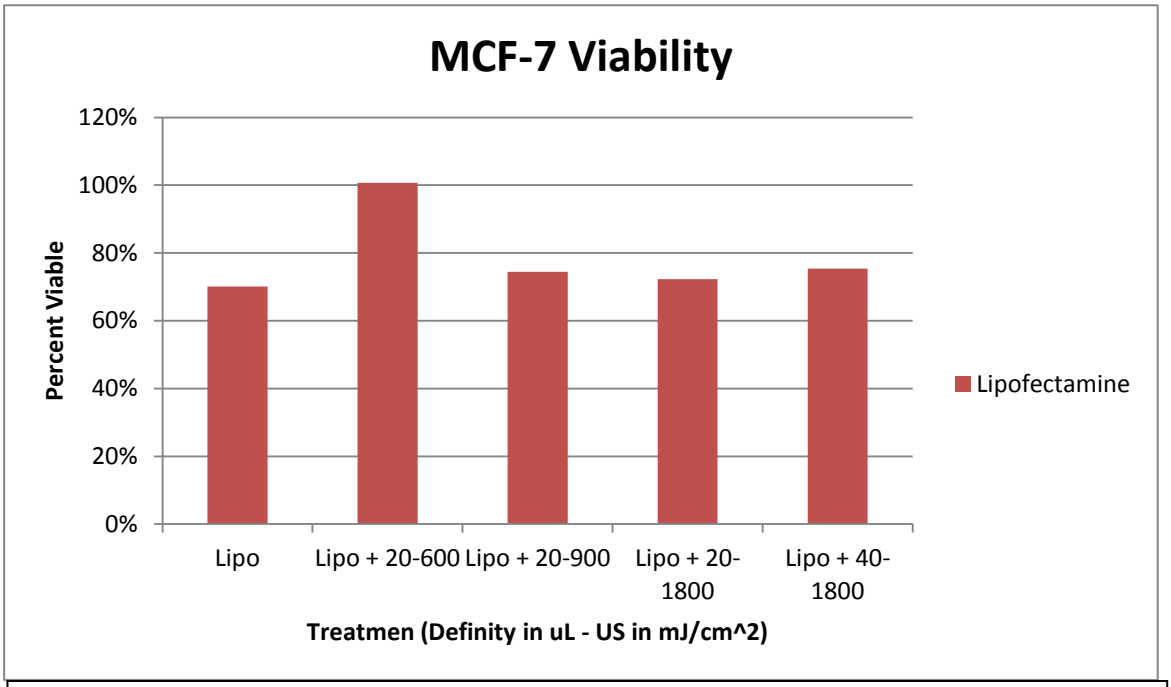

Figure 4.22 - The percent of viable suspended MCF-7 cells transfected 72 hours after Lipofectamine 2000 was added to cells before they were sonoporated together with Definity.

The results in figure 4.21 do not show any synergistic properties; sonoporation may decrease the transfection rate. The finding concurred with Reslan's paper in 2010 where she reported that combining sonoporation and lipofectin, another liposome mediated transfection reagent, did not enhance transfection [98]. More importantly, Lipofectamine is a relatively toxic transfection reagent. The cell viability was only around 70%. The results were not encouraging, and the results were not repeated.

4.3.2 - Self-Made Phosphoipid Coated Hydrated Microbubbles

Definity™ is not the only commercial microbubbles, but other ultrasound contrast agents are not obtainable in Canada. To test the possibility of using other microbubbles for sonoporation, a new microbubble formulation was developed. The self-developed microbubbles consist of a phospholipid shell held together by surfactant with an atmospheric air gas core. The protocol for synthesis is explained in Chapter 3. The formulation for the microbubbles was developed by Dr. Gu, and Miss Aditi Ganji helped with the synthesis.

Many combinations of phospholipids and surfactant were tested; they were selected for stability, sizes, uniformities, and the ability to be cavitated by inertial cavitation. The most successful microbubble composed of DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), Cremophor EL and Glycerol. Details of the ratio and activation methods are recorded in Chapter 3. Images of the microbubbles inside hemocytometer have been captured; Figure 4.23 shows both images of before and after ultrasound treatment. The ultrasound which used to cavitate these microbubbles came from the 1.5MHz SonaCell at 0.2W/cm^2, 20% Duty Cycle and 1Hz repetition rate.

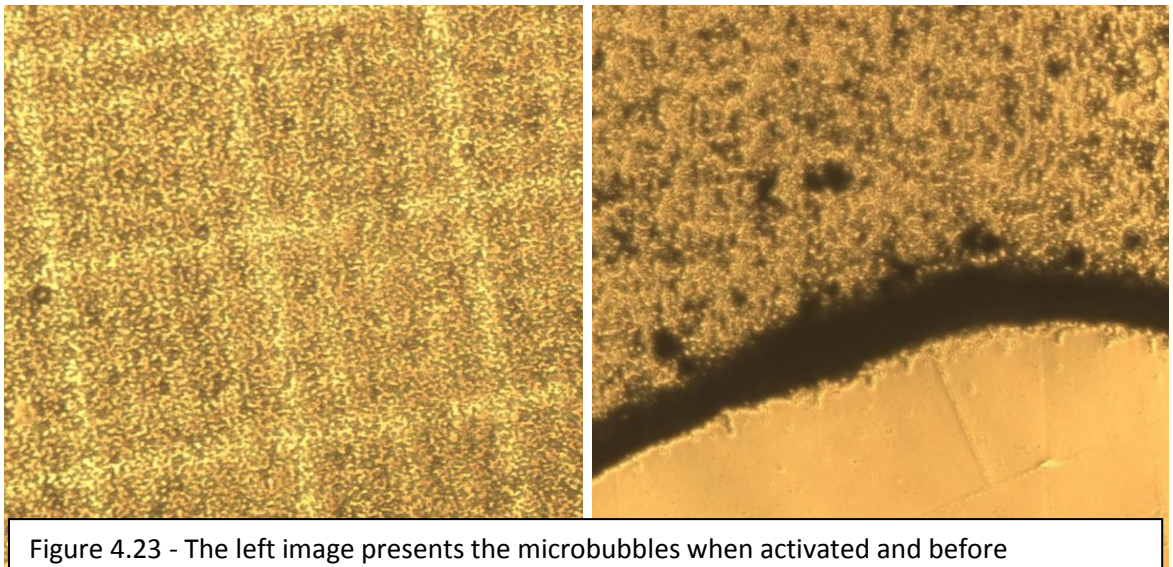

Figure 4.23 - The left image presents the microbubbles when activated and before ultrasound treatment. The right image represents microbubbles after ultrasound. The scales of on both of the images are the same; the sides of each box represent 250um.

The image on the left in Figure 4.23 shows that the microbubbles have a uniform size of around 10um in diameter. The microscope used to capture these images did not have stronger objective lens, hence, a more accurate measurement for the diameter for the population is not possible. The concentration of bubbles cannot be estimated also due to the low magnification. A multisizer would be useful to identify the microbubbles' size and concentration, but such equipment was not readily available. The images prove that the microbubbles were affected by ultrasound. The microbubbles were dispensed into the hemocytometer, and ultrasound was applied from the bottom of the hemocytometer. The hemocytometer had 30% transmission efficiency. The ultrasound was turned on for a short moment; the ultrasound was stopped as soon as the milky whiteness of the microbubbles started to disappear. The image on the right in Figure 4.23 does not share the same pattern of shinny spheres on transparent background as the image on the right, but become a dark puddle of phospholipid. The images prove the microbubbles are physically affected by ultrasound. This change in physical appearance is believed to be brought about by inertial cavitations.

The suspended MCF-7 cells were used to test the DSPC hydrated microbubbles's delivering ability and toxicity. Concentration of microbubbles tested were: 20,40,80,120 and 160uL per dish with $3x10^5$ cells in 350uL of medium. Since the microbubbles are still in development, they were not tested with GFP plasmid; rather they were tested with FITC-Dextran. As mention in previous chapters, FITC-Dextran is cheaper and can help detect changes in permeability of cell membrane. Therefore, the delivery of FITC-Dextran is suitable for the purpose of this experiment to verify if the phospholipid-coated hydrated microbubble can facilitate preopening on cell membrane. 1uL of FITC-Dextran dissolved in PBS, at a concentration of 5ug per mL, was added to each sample before ultrasound was applied. The ultrasound was

generated by the 1.5MHz SonaCell box at 0.2W/cm^2, 20% Duty Cycle for 30 seconds, or 1200mJ/cm^2 of energy deposition. The ultrasound was applied in exact manner as sonoporation experiments with Definity. A sample of suspended MCF-7 cells with 80uL of hydrated microbubbles without of ultrasound was used to determine the background toxicity of the microbubbles. MTT assay and flow cytometry were tested 24 hours after the experiment; the results were displayed in figure 4.24 and 4.25.

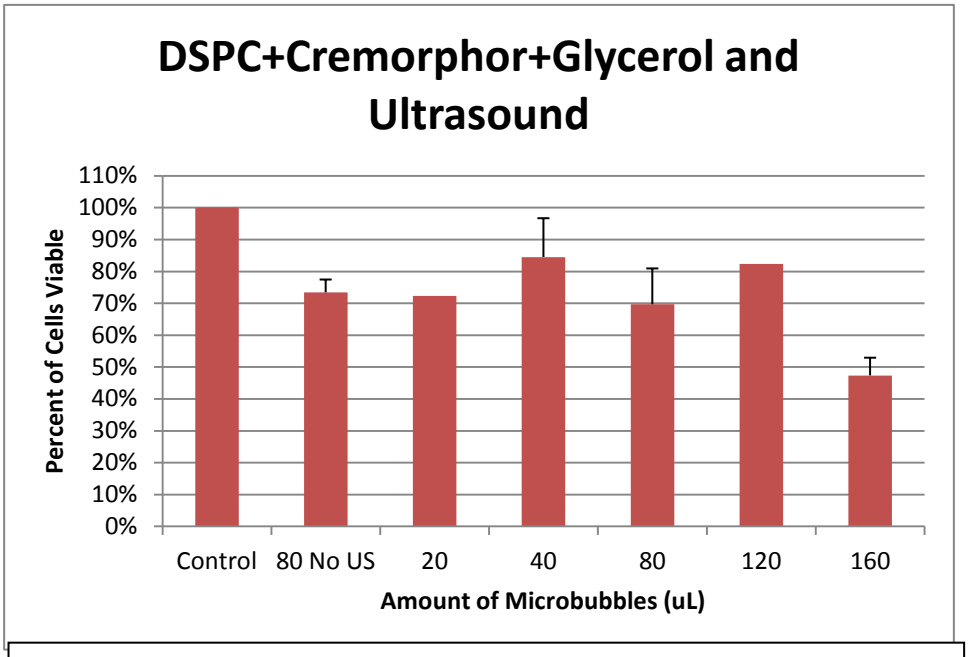

Figure 4.24 - The viability of cells 24 hours after they were treated with ultrasound and DSPC+Cremorphor+Glycerol microbubbles.

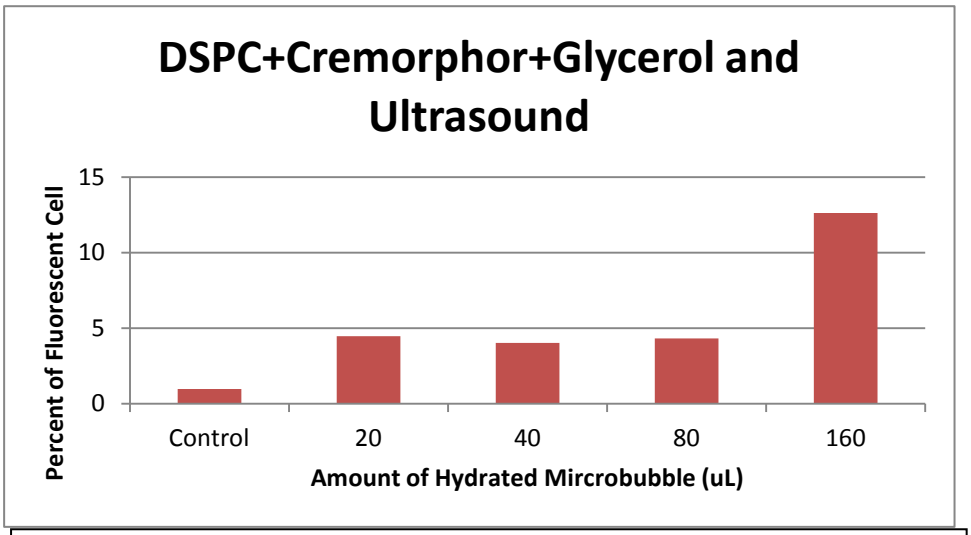

Figure 4.25 - The delivery rate of cells 24 hours after they were treated with ultrasound, FITC-Dextran and DSPC+Cremorphor+Glycerol microbubbles.

From figure 4.24, the data suggest that the hyrdated microbubbles were somewhat toxic to the cells. The samples without ultrasound treatment, they have approximately 30% cell death. This is at the edge of what is believed to be the limit of safe transfection methods. With ultrasound, the viability remains the same; sample with 160uL of hydrated microbubbles was an exception. This may have caused by the excessive amount of microbubbles used. The cells did not reattach themselves to the bottom of their dish 24 hours later for those experiment samples. The bottom of the dish was covered with a greasy film, suspected to be the remnant of the cavitated microbubbles. The DSPC and Glycerol film could have prevented the MCF-7 cells from reattaching and proliferating. In figure 4.24, the samples did not have transfection greater than 5% except for the sample with 160uL of hydrated microbubbles, which have 15% delivery rate.

The formation of the microbubbles used in this experiment is still under development. Substitute with other less toxic surfactant is currently under development. However, the results show that it is possible to perform microbubbles with self-developed

microbubbles. The application of these microbubbles can impact the field of sonoporation in the future.

4.3.3 - SonaCell Proliferation Delivery

SonaCell was originally used for stem cell proliferation. In previous experiments, it was confirmed that SonaCell can proliferate MCF-7 and KG-1 cells effectively. This proliferation property of SonaCell may aid sonoporation by increasing the amount of collateral damage of sonoporation cells can withstand. To test this property, KG-1 cells were used. $7.5x10^5$ KG-1 cells were placed in 350uL of medium with 20uL of Definity™. In this experiment, 5uL of FITC-Dextran in PBS at concentration of 5mg/mL was used instead of GFP plasmid in this experiment. The cells were the collected 24 hours after the treatment. The ultrasound used for this experiment was generated by the SonaCell box with 1.5MHz transducer. The protocol to set-up the experiment is the same a previous experiments with cells placed in the middle of a mounted 35mm dish. However, in this experiment, the samples were left in ultrasound field for 10 minutes. This extended period of sonication is to emulate the protocol to stimulate stem cell proliferation; stem cells were sonicated at $150mW/cm^2$ for 10 minutes. The extended duration of sonication increases the energy deposition. Lower ultrasound intensities were added to test to help find the safe range of ultrasound intensity; the intensities tested were: 0, 10, 20, 40, 80, 120, 160 and $200mJ/cm^2$.

MTT assay and flow cyctometery was used to analyze the samples for viability and delivery rate; the results are shown 4.25 and 4.26.

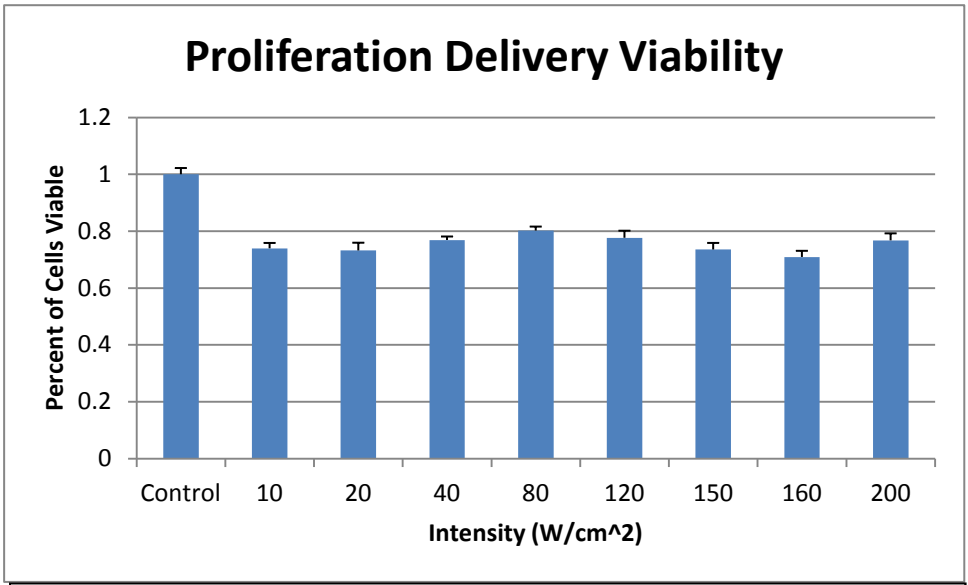

Figure 4.26 - The viability of KG-1 cells 24 hours after treated with 10 minutes of ultrasound and 20uL of Definity.

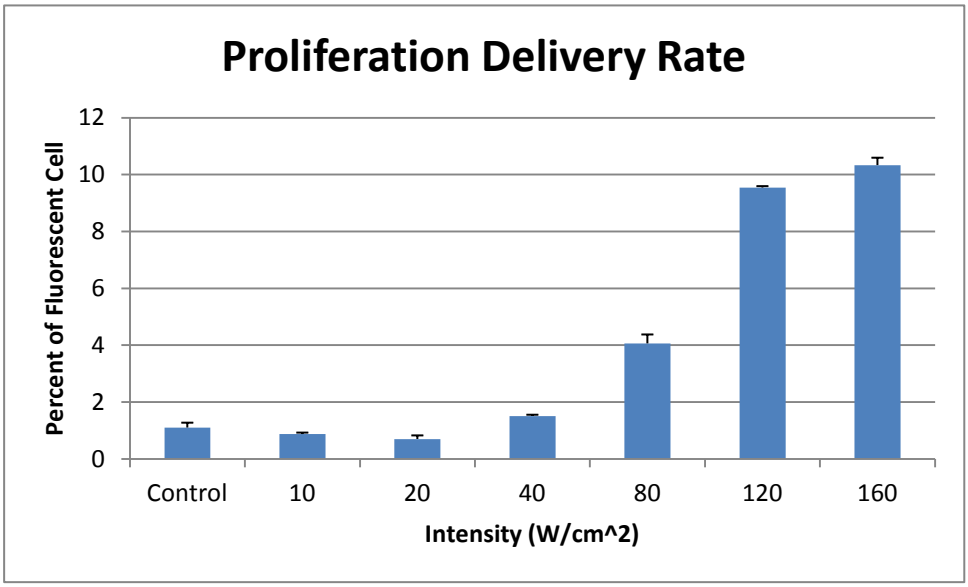

Figure 4.27 - The delivery rate of KG-1 cells 24 hours after treated with 10 minute of ultrasound with 20uL of Definity and FITC-Dextran.

The results were not comparable to previous experiments on KG-1 with GFP plasmid as the collection time and transfection marker used were different. The previous experiment was collected 72 hours to allow GFP to be translated, but FITC-Dextran does not require the protein expression processes and could be analyzed without further culturing. A longer incubation period would give cell population more time for cell population to rebound, leading to higher viability. Since the transfection markers are different in the two experiments, the percentage of cell with fluorescent cannot be compared either.

Figure 4.26 shows that the viability of cells were above 70% for all samples. This is surprising as these conditions had more ultrasound energy deposited in them than pervious experiments. The MTT assay suggests that the KG-1 cells can withstand extended period of low intensity sonication. The viability may rebound quickly in the next 24 to 48 hours, which will lead to higher cell viability readings as seen in other experiment.

As for delivery ability, it is possible to deliver FITC-Dextran to KG-1 cell using the proliferation protocol. Figure 4.27 confirms that there is as much as 10% delivery rate from samples sonicated with 160 mW/cm^2. There are two more things which can be inferred from the trend displayed in Figure 4.27. The delivery rates for samples with 10, 20 and 40 mJ/cm^2 ultrasound are below 2%, which are not significantly higher than the controls. There was an increase in delivery rate for 80 mJ/cm^2 samples to about 4%. As intensity increased to 120mJ/cm^2, the delivery rate rises to 9%, but the increase in delivery rate plateaus at 160mJ/cm^2. The first part of the trend suggests that Definity™ is not actively facilitating sonoporation when sonicated with low intensity ultrasound even for extended duration; Definity™ microbubbles starts to become active after 40mJ/cm^2. Without reaching a certain threshold, the microbubbles will not bring about delivery, as seen in samples sonicated with 40 mJ/cm^2 and below. The second part of the trend suggests that the amount of Definity™ in each dish limits the delivery

rate. This concept is reassured previous understanding: Definity™ is the determinant factor for causing deaths in sonoporation. Higher volume of Definity™, more inertial cavitations occurs, causing more pores to be created on the cell membrane, resulting in higher permeability and cell death.

4.3.4 - Multiple Session Sonoporation

Another way to exploit the proliferation ability of the SonalCell was to apply sonoporation multiple times. Unlike the Excel UltraMax commerical ultrasound box, SonaCells preserves viability of cell after sonoporation, hence allowing the cells to go through another cycle of sonoporation the next day. By increasing the number of exposures to sonoporation, it should increasing the overall numbers of cells sonoporated.

Secondly, the multiple session sonoporation experiment includes the concept of cell selection. It was believed that the huge variability between repeats of the same experiment is partly due to the differences in population's state; the amount of cells in S phase, G phase, or M phase relative to each other. When the cells were harvested, cells may not always be growing in the same conditions as previous batches. Their concentration might be different, the amount of nutrients available might be different or the time of the day may have been different. These differences in growing and harvesting conditions will change the population's cell cycle ratio; there might be more cells in their M phase when a population is in logarithmic growth phase as compared to a population in their stationary phase. This difference in amount of cells at various cell cycle may lead to the variation in experiments. It is possible that ultrasound helps reset the cycle in cells, hence allowing to proliferate more efficiently. Under such an assumption, the first session in multiple session sonoporation may act as a synchronization signal for the cells, such that the population of cells are in unity when sonoporated for the second time, stabilizing the results.

The protocol for multiple session sonoporation is similar to the protocol to transfect KG-1 cells with GFP plasmid, Definity™ and ultrasound. However, before each succession session of sonoication, samples were collected, centrifuged and resuspended in 350uL of stock medium. Another session of sonoporation was performed after GFP plasmid and Definity™ were added to

the resuspended cells. 1 hour after each sonoporation session, 650uL of serum rich medium was added to the samples and allowed to grow for 24 hours in the incubator.

Two sonoporation conditions for multiple sonoporation were chosen, one with energy deposition of 1800mJ/cm^2, the other with 600mJ/cm^2. 1800mJ/cm^2 was generated by 1.5MHz SonaCell box with 20% Duty Cycle and 0.2W/cm^2 for 45 seconds, while the 600mJ/cm^2 was generated with 10% Duty Cycle and 0. 2W/cm^2 for 30 seconds. Both conditions had 20uL of Definity™ added to facilitate sonoporation. Each condition has six different treatments as listed on Table 4.7; not all treatments require GFP plasmid or sonoporation daily. The samples were treated for 3 days, 24 hours in between each treatment. The cells were analyzed with MTS assay and flow cytometery 72 hours after the last treatment; the results are compared in figure 4.28 and 4.28.

Treatment	Day 1	Day 2	Day 3
1	GFP + US + DEFINITY	GFP + US + DEFINITY	GFP + US + DEFINITY
2	GFP +US + DEFINITY	US + DEFINITY only	GFP + US + DEFINITY
3	GFP +US + DEFINITY	SKIP	GFP + US + DEFINITY
4	US + DEFINITY only	GFP + US + DEFINITY	GFP + US + DEFINITY
5	US + DEFINITY only	US + DEFINITY only	GFP + US + DEFINITY
6	US + DEFINITY only	SKIP	GFP + US + DEFINITY

Table 4.7 - The layout of multiple session sonoporation experiment. GFP represents Green Fluorescent Protein plasmid was added to the sample; US represents that ultrasound was used on the sample; DEFINITY represents 20uL of microbubbles were used on the sample.

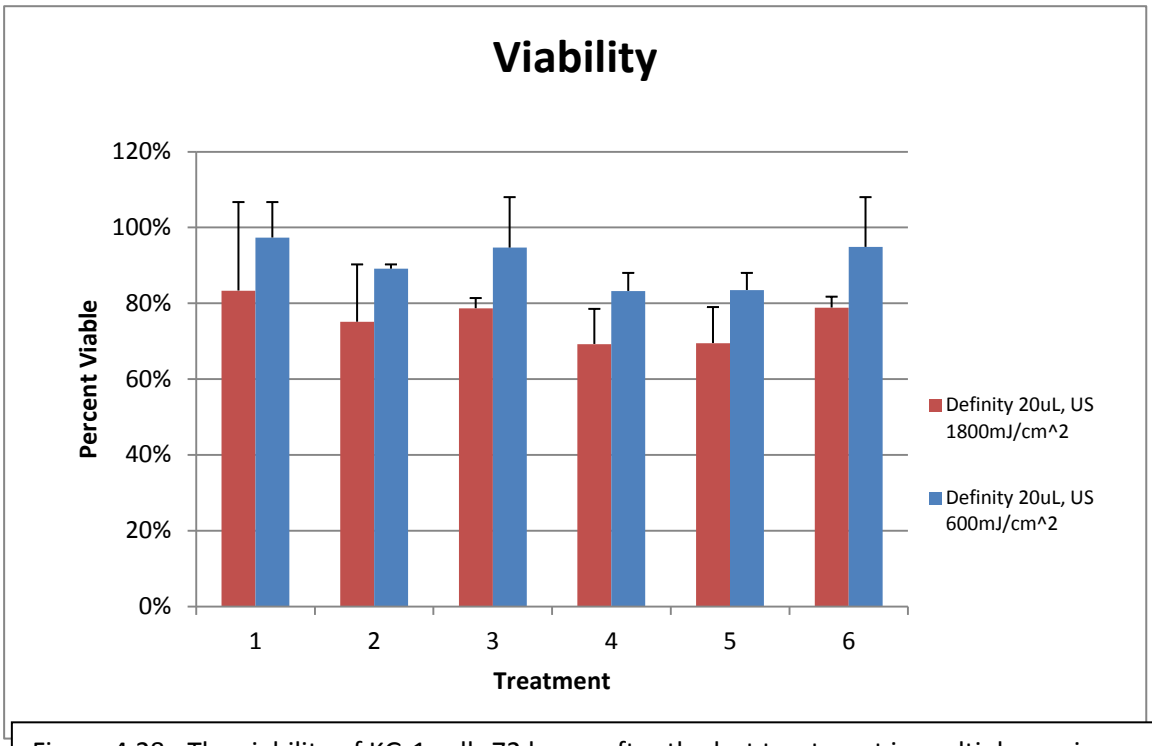

Figure 4.28 - The viability of KG-1 cells 72 hours after the last treatment in multiple session sonoporation experiment.

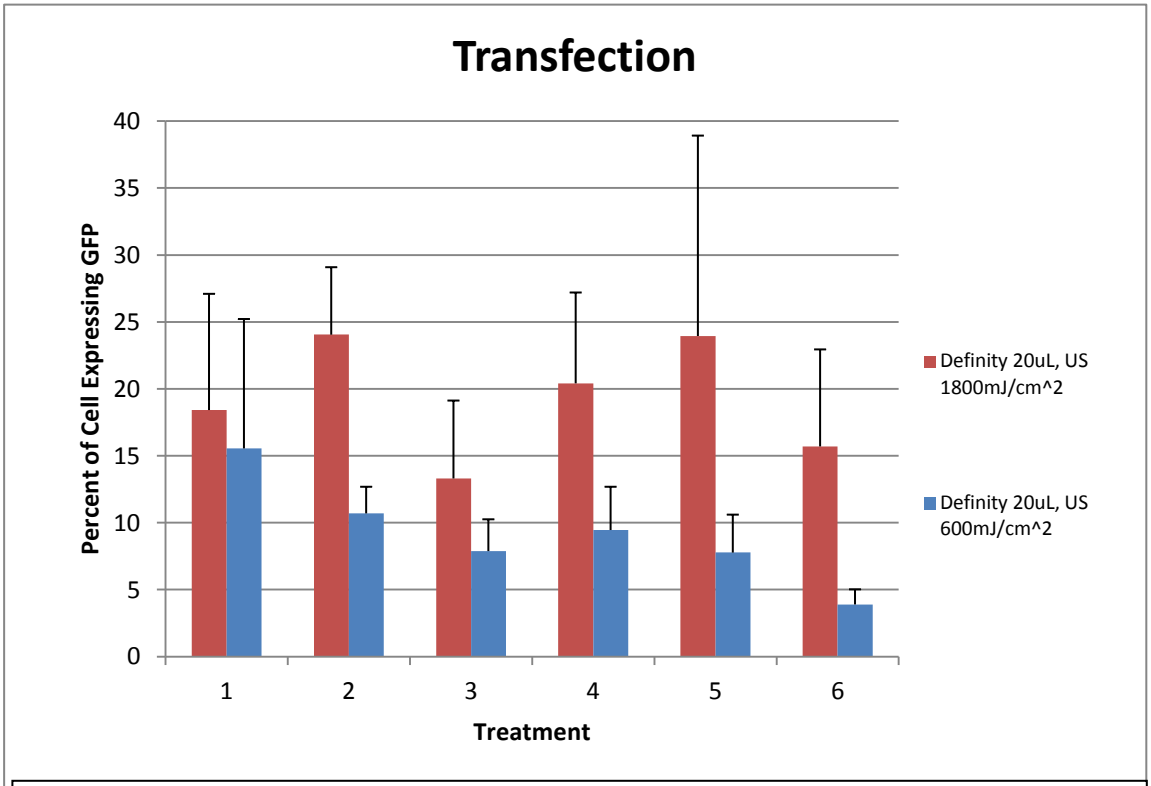

Figure 4.29 - The transfection rate of KG-1 cells 72 hours after the last treatment in multiple session sonoporation experiment.

The results on figure 4.28 confirm that the KG-1 cells can withstand three successive sonoporation treatments and the viability rebounded to above 70% for all samples. The transfection results on figure 4.29 are more encouraging. The highest transfection rate was able to reach 24% with viability of 70%. This is a significant jump from 10% transfection rate from single session. The results prove the multiple session sonoporation to be a feasible improvement to sonoporation in transfecting difficult-to-transfect-cells.

Chapter 5
Conclusion and Discussion

Sonoporation is not a perfect delivery method . Just as other physical delivery methods, such as electroporation, the shortcomings prevent it from being adopted as a mainstream technique. The greatest challenge to overcome for sonoporation is the adverse effects on cell viability after the treatment. Sonoporation opens the cell membrane with mechanical force, allowing foreign materials to enter. In an ideal situation, the pore formation is reversible, where cell membrane repairs itself and seals the pore. The cell membrane reparations occur within five seconds after pore is formed [99,100]. The pores' sizes are approximately 110nm in radius with a standard deviation of 40nm [100]. However, generating pores greater than those stated in literatures is possible and can lead to irreparable damages on the cell membrane, which causes cell death. Therefore, controlling cellular damages during the sonoporation process is so critical, such that pores are formed and repaired within short amount of time.

Experimental results in this thesis confirmed many known trends and challenges of sonoporation. Furthermore, new techniques for sonoporation were explored and evaluated. The details of the results are described in Chapter 4. The impacts of these findings and difficulties will help future scientific research for ultrasound mediated delivery.

5.1 – Trends

5.1.1 – Cell Viability

5.1.1.1 - Ultrasound

For the experiments conducted in this thesis, a generalized unit of ultrasound was used. Energy deposition, measured in energy per unit area ($\frac{J}{m^2}$), was used to describe the amount of

energy cells received from ultrasound waves. This unit summarizes the intensity, duty cycle, and duration of ultrasound, as energy deposition is defined as:

$$Energy\ Deposition = \ Time * \ Intensity * \ Duty\ cycle = s * \frac{J/s}{cm^2} * \% = \frac{J}{cm^2}$$

This unit is a good predictor of the cell viability. Higher energy deposition will cause more cell death. This is expected as higher energy will increase the number of inertial cavitations of the ultrasound contrast agents used to facilitate pore opening, leading to more severe damages on cell membrane.

However, there are many ways to generate the same energy deposition; the three variables of energy deposition can compensate each other's changes. In the experiments it was apparent that some parameters are not suitable for sonoporation. The values of these parameters may not be the same in all ultrasound machines, as their frequency, transducer size and repetition rate may vary, but the trends are similar.

The results suggest that the best way was to increase energy deposition is by increasing ultrasound duration. Compared to changing duty cycle and intensity, an increase in ultrasound duration does not dramatically decrease cell viability. However, giving excess energy through extending duration can still cause cell death.

Changing duty cycle can lead to a change in cell viability. Duty cycle influences how often the ultrasound waves hit the sample. While holding energy deposition constant, increasing duty cycle will increase cell death. Susceptibility to different duty cycle varies between cell lines. However, it was apparent that pulsed ultrasound waves preserves cell viability better than continuous ultrasound wave (100% duty cycle).

Intensity determines how strong each pulse of ultrasound waves hits the sample. In general, increasing intensity causes more microbubbles to undergo inertial cavitation, leading to more severe damages and more cell death. This trend holds true with conditions restricted by

ultrasound contrast agents at both extremities of intensity. The amount of cell death at high intensity is limited by the amount of ultrasound contrast agents in the sample. At high energy, cell death plateaus. Since most of the microbubbles were already bursted at a lower intensity ultrasound, increasing intensity will not trigger more inertial cavitation. Microbubbles will not undergo inertial cavitation unless the energy delivered by ultrasound waves surpasses the threshold energy of inertial cavitation, thereby limiting cell death at lower ultrasound intensities. Therefore, the trend is true if intensity is above the threshold for inertial cavitation of the ultrasound contrast agents.

5.1.1.2 – Ultrasound Contrast Agent

Ultrasound contrast agents are small bubbles (<7μm) with gaseous core used to facilitate sonoporation. They are the key parameters in controlling cell viability. The number of microbubbles, the inertial cavitation thresholds, and their proximity to cells determine the cellular damage caused in samples.

The number of microbubbles per sample will restrict the maximum number of inertial cavitations which can occur in a treatment. More inertial cavitations will generate more damages and cause more cell deaths. The number of inertial cavitations is determined by inherent size distribution of the microbubble population, inertial cavitation threshold of the bubbles, which is determined by microbubbles' chemical composition, and the intensity of incident ultrasound pulses. The size of microbubble will affect its inertial cavitation threshold and resonance frequency. Inertial cavitation threshold is the minimum amount of oscillation energy required to burst the microbubble. Resonance frequency affects the efficiency in converting ultrasound energy into oscillation energy; increasing incident ultrasound intensity can compensate for low conversion efficiency. If a microbubble receives oscillation energy

greater than the energy threshold required to burst it, inertial cavitations may occur. The microbubbles are not uniform in sizes, but follow certain distribution. It is possible for incident ultrasound to cause inertial cavitation for some microbubbles while leaving the rest of the microbubbles intact. Viability is adjusted by controlling the number of inertial cavitations in the sample.

Distance between microbubbles and cells affect the severity of sonoporation damages. Inertial cavitation will generate shockwave and radical, which are damaging to cells. However, these effects are short lived and only affect a small volume surrounding the cavitation. It was noted in paper by Le Gac in 2007, that if cells were located at a distance twice the diameter of the microbubble, the cavitation will not have any effects on the cells [101]. Since the ultrasound contrast agents are usually smaller than 7µm in diameter, cell located 14µm away from a microbubble might not be affected by sonoporation. The results in this thesis tells us that Definity™ might not be evenly dispersed in the medium, but rather they were attracted to cells. This suggest that the concentration of microbubbles within the sample is not the determinant factor, rather, the ratio between cell number and number of Definity™ microbubbles will be a better predictor of cell death in sonoporation.

Results in this thesis suggest that SonaCell producing 1.5MHz ultrasound with 1Hz repetition frequency helped increase cell proliferation. This trend was shown in suspended MCF-7 and KG-1 cells. A 30 second treatment will already show the proliferation function of the ultrasound box. This proliferation ability of the SonaCell was exploited to increase sonoporation efficiency.

5.1.2 –Transfection and Delivery

Ultrasound is the source of energy for sonoporation. Controlling ultrasound parameters can help balance cell viability and rate of sonoporation. In the past, researchers believed that stronger ultrasound intensity, or more energy deposition will cause more inertial cavitation, which lead to more cells being sonoporated. However, Forbes' research and the results in this thesis suggest that higher energy does not necessary result in higher delivery efficiency [94]. Efficiency increases as energy further increases at lower energy until an optimal point, and then the efficiency drops as energy increases. This phenomenon was believed to be contributed by the participation of stable cavitation or microstreaming. At higher energy, more microbubbles were bursted by inertial cavitation and fewer microbubbles were left for stable cavitation. A decrease in number of microbubbles for stable cavitation led to less efficient delivery and transfection. Therefore, optimal sonoporation does not solely depend on energy of the ultrasound, but dependent on the ratio of microbubbles in stable cavitation to inertial cavitation. In future experiments, using two ultrasound contrast agents of different sizes may prove to be advantageous; one type will undergo inertial cavitation while the other participate in stable cavitation. This will theoretically lead to higher sonoporation.

Green Florescent Protein and FITC-Dextran were used as transfection and delivery markers. Their presence in cells of sonoporated samples indicated the cells' membrane permeability were changed during ultrasound treatment. The concentration of the plasmid or the macromolecule in the medium will influence the amount of markers present in the cell and the fluorescent intensity. Flow cytometry results from experiments using different concentrations of markers cannot be directly compared. Expression level of Green Fluorescent Proteins plasmid will affect the analysis as well. Therefore, concentration of GFP plasmid and FITC-Dextran should be held constant.

In the experiments, it was determined that higher concentration of GFP plasmid will lead to higher transfection rate. However, there is a diminishing return in the amount of plasmid placed in each sample. Since plasmid production is expensive and time consuming, an adequate amount of GPF plasmid will keep the cost low, yet indicate the efficiency of transfection. FITC-Dextran is a low cost sonoporation marker, but adequate amount of FITC-Dextran is critical for accurate flow cytometry analysis. FITC-Dextran has high affinity to cell membrane and is difficult to wash off. Excessive amount of FITC-Dextran can lead to false positive results of sonoporation. Due to the high sensitivity of flow cytometry, small amount of FITC-Dextran is enough to help quantize permeability of cells. To view positive results of sonoporation under confocal microscope, a much higher amount of FITC-Dextran is required as confocal microscope is less sensitive.

5.1.3 – Cellular Specificity

The results of this thesis also suggest that sonoporation is a cell-line specific technique. Every cell line reacts differently to various parameters of sonoporation. Some cell lines can withstand more sonoporation damages and some cell lines can recover quicker. Different property of cell line alters the susceptibility and effectiveness of sonoporation. Even the same cell line can react differently to sonoporation when treated in adhesion or in suspension, as demonstrated with MCF-7 in this thesis. Therefore, optimization is necessary for each type of cell line by varying ultrasound intensity, duty cycle, duration, frequency, repetition rate, amount of marker, amount of medium, number of cells, type of ultrasound contrast agents, and amount of ultrasound. Empirical tests should be performed to determine the cell line's susceptibility to sonoporation.

5.2 - Reflection on New Technique

5.2.1 - Synergy with Lipofectamine 2000

The results did not show that cells incubated with lipofectamine while sonoporated resulted in an increase of transfection. The greatest drawback of this technique was the high toxicity of Lipofecatimine 2000. The chemical was lowered cell viability by 30% without ultrasound. The chemical transfection is too toxic for the purpose of this thesis. The other chemical transfection agents did not successfully transfect suspension cells. The inability and the toxicity of chemical transfection agents needs to be overcame before the synergistic technique can be further developed.

Recently, Mr. Peng Xu, a lab-mate and graduate student from the Biomedical Engineering Department of University of Alberta, had shown that suspended KG-1 cells can be transfected with considerable success with IBAfect. He was able to delivery CXCR4 plasmid to 30% of KG-1 cells in his experiments. His procedure required KG-1 cells to be attached to the bottom of the 35mm culturing dish with the aid of Poly-L-lysine. The success of his experiment with IBAfect on KG-1 cells suggest that IBAfect or other chemical transfection agents may increase their transfection ability by having the suspension cells attached to the bottom of the culturing dish. Xu's technique increases transfection efficiency of other less toxic chemical transfect agents, hence opening the opportunities for other chemical transfection reagent to synergistically transfect cells with sonoporation.

5.2.2 - Self-Developed Phospholipid Microbubbles

The DSPC+Cremophor+Glycerol microbubbles proved to be capable in aiding delivery of FITC-Dextran in sonoporation, as a substitute for Definity™. However, there are plenty of drawbacks and experimentations required to refine this technique.

The greatest drawback is the toxicity of the microbubbles. Results suggest that more bubbles will result in higher efficiency of delivery, but the concentration of bubbles become too toxic to cells at 80uL. The toxicity of the microbubble limits its effeciency to deliver FITC-Dextran. Therefore, if more microbubbles can be used, higher delivery rate can be achieved in both adhesion and suspension cells. Using a less toxic surfactant, such as Tween-80, may help decrease its toxicity. Furthermore, a lower concentration of glycerol or replacing culturing medium couple of hours after the experiment may help suspended MCF-7 cells to reattach to the bottom of the culturing dish and allow them to proliferate. In addition, a transfection using GFP plasmid as sonoporation marker with an increased incubation and growing time before analysis in MTT assay and flow cytometry would be necessary to directly compare its efficiency with other techniques.

It is also important to understand the mechanism of the microbubbles. It might be possible that the self-developed microbubble can increase transfection rate more than the commercial ones. Since the shell consists of phospholipid, the microbubbles can participate in endocytosis similar to lipofectamine and other liposome based chemical transfection agents. Preliminary results, not included in this thesis, suggested that endocytosis was a possible mechanism for these phospholipid microbubbles to deliver the FITC-Dextran into cells. However, microscope confirmed the physical change after ultrasound treatment suggests that sonoporation should have taken place. A synergistic of the two mechanisms is possible. The confirmation of mechanism requires further testing.

In the future, a spatial specific function of these less toxic, duo-delivery-mechanism microbubbles can be added. The shell of the phospolipid microbubbles can be chemically modified and be linked to cell targeting macromolecules. These microbubbles will attach themselves to a particular type of cell in the body, enabling the localized delivery of drugs and

genes via sonoporation to those cells and in a particular area. This would decrease toxicity by minimizing the amount of microbubbles used. Further development may allow the drug or plasmid to be linked to the membrane or be encapsulated within the bubble. This enhancement would aid in reducing the cost and the side effects of drugs or genes delivered in sonoporation-mediated therapy for in vivo treatments.

There are plenty of potentials for this technique of using self-developed microbubbles rather than using commercially available ultrasound contrast agents. However, extensive research and development are still required before they can reach their full potential.

5.2.3 - SonaCell Proliferation Delivery

The original use of SonaCell was to increase proliferation rate of teeth and stem cells. The proliferation ultrasound machine shown to be capable of performing sonoporation while providing its proliferation benefits to treated samples in various experiments. Sonoporation procedures require a short ultrasound application with ultrasound contrast agent, while proliferation procedures require a longer ultrasound application without ultrasound contrast agent. It was thought that higher viability can be achieved if the sonoporated cells were left in the ultrasound field for ten minutes, same as stem cells proliferation protocol.

The results were promising. The cell viability did not drop drastically with higher amount of energy deposited into the samples. Also, FITC-Dextran confirmed that this technique was able to change permeability of the KG-1 cells' membrane. The rate was able to reach 10% with 160mW/cm^2; higher delivery rate may be possible with the use of a higher intensity ultrasound. GFP plasmid should be used for sonoporation to directly compare its efficiency with other techniques; incubation and growing time will have to be increased before MTS assay and flow cytometry analysis are performed. Longer growing time will lead to a higher viability, as cells will

have more time to recover from the treatment, which would allow higher energy or more Definity™ microbubbles to be used for sonoporation, which may lead to a higher transfection rate. This will hopefully lead to a new sonoporation protocol and a standard for single session sonoporation. This proved the possibility to perform sonoporation on cells while they are being proliferated with the SonaCell, minimizing the procedure required to prepare stem cell implantation in clinical studies.

5.2.4 - Multiple Session Sonoporation

A relatively easy way to increase transfection rate would be to administer sonoporation to samples more than once. This technique requires more GFP plasmid and Definity™. The results were not very stable, but it shows that higher transfection can be made without compromising cell viability. More experiments need to be performed to stabilize the results and confirm with statistical confidence that this technique works better than single session sonoporation.

The results in the multiple session sonoporation also show a possibility that there is a provisional effects of ultrasound. One hypothesis of the experiment was that ultrasound may be possible to help reset cell cycle resulting in more stabilized transfection rate. The experiment did not have stable transfection results, ruling out the hypothesis. However, samples that had an ultrasound treatment with Definity™, with or without GFP plasmid, the day before they were sonoporated with GFP plasmid, resulted in a higher transfection rate. This trend was not seen for samples which were sonicated 48 hours before sonoporation with GFP. There is a provision effects shown in the experiment, but is present for only a short amount of time, less than 48 hours. Further studies are required to understand on prove the effects and how to use provisional ultrasound in sonoporation to increase transfection efficiency.

Overall, multiple session of sonoporation proves to be a promising technique. The cost of having multiple sonoporation would be the prominent drawback of this technique. However, it is relatively effective in delivering GFP plasmid into KG-1 cells, a hard-to-transfect cell line. Further investigation of provisional ultrasound may prove to be an alternative protocol for multiple-session sonoporation reducing the amount of GPF required while achieving similar level of transfection rate and preserving cell viability.

5.3 - Experienced Difficulties and Complications

5.3.1 - Ultrasound Beam Profile Analysis

Throughout this thesis, ultrasound beam profile was not measured. It is uncertain whether the ultrasound beam was wide, narrow or focused. This may lead to an incorrect measurement of intensity with the power meter if the power meter's sensor was smaller than the beam size. Since transducer determines the ultrasound beam profile, changing transducer will change the profile of the beam generated. This will cause problems when other researchers try to repeat the same experiment. This might lead to unrepeatable experiments. There was no equipment available to measure the beam profile; a hydrophone is required as power meter is not spatial sensitive.

The lack of record for ultrasound transducer had wasted plenty of my time. The original transducer was purchased from American Peizo Ceramics (APC), but it burnt out after a year of experiment. The new SonaCell transducer was not from APC, therefore, there might have been a difference in beam profile. Some of the previous experiments done with the APC transducer cannot be repeated. It was necessary to optimize the individual cell lines over again. Therefore, some of experiment results were not repeated with enough times, leading to a large error bar on graphs. The new transducer still produces 1.5MHz ultrasound, but the quality of ultrasound might not be the same. Among the same batch and shipment, the new transducers had various efficiencies; with constant voltage applied to the transducers, some produced more intense ultrasound, some produced less. This inconsistency within the same batch suggests that their beam profile might vary dramatically between individual transducers of the same brand. Since there is a discrepancy in the replaced transducer and the original APC transducer, direct comparison was impossible, hence, future experiments used FITC-Dextran instead of GFP plasmid as delivery markers.

5.3.2 - GFP Plasmid Production

There are many potential problems with using plasmid as a transfection marker. The advantage of using plasmid is that it would simulate all the steps required for a successful delivery in gene therapy. However, there are complications in comparing between literatures. Some literatures used other plasmid coding for fluorescent protein, such as luciferase. Even for literatures using GFP-protein, many do not state the company or the expression level of the plasmid; a difference in expression level could have led to different interpretation in flow cytomey. This is a problem because flow cytomety distinguish cells in relative term. Higher expression level plasmid could cause some weakly transfected cells to express more GFP and be interpreted as a positive result; a lower expression level plasmid would have expressed much less, leading to an interpretation of a negative result. This discrepancy when using different plasmid of expression level will lead to problems when comparing literatures.

Furthermore, even when using the same plasmid, the quality may differ from batch to batch. The purity of the GFP plasmid will be different. This might have been one of the reasons for the large error bars on transfection rate in experiments of this thesis. When adding GFP plasmid to samples for experiments, a certain volume was pipetted according to calculations; weight divided by concentration (ng/uL). The concentration does not describe the weight of plasmid per unit volume of solution but refers to the weight for total genetic material per unit volume, both plasmid and other impurities. Therefore, the actual amount of plasmids used in each experiment may have been different. In the future, a standard for impurities should be set or be accounted for in calculations.

GFP plasmid expansion is time consuming and expensive. Moreover, there are plenty of complications in GFP expression. Therefore, it is understandable why some researchers, including this thesis have chosen FITC-Dextran as an alternative sonoporation marker.

5.3.3 - Flow Cytometry

As emphasized previously, flow cytometry is a technique which measures and records the relative fluorescent level of individual cell. There are many different ways to analyze flow cytometry, and each will lead to a different conclusion about the sample. Some experimental techniques will have small fluorescent differences in the population's change, for which an overlay technique should be used. The overlay technique accounts for the shift of the fluorescent intensity of the population; the number of cells in sample expressing fluorescent intensity higher than control sample's mode minus the number of cells in control sample expressing higher than its mode is the number of positive cell. However, this might not be a suitable method to analyze GFP transfection. The alternative technique to analyze flow cytometry results would be characterize the normal distribution of the controlled population and consider cells exhibiting intensity higher than the maximum fluorescent intensity of controlled group to be transfected cells. This technique would be less sensitive to cell exhibiting low fluorescent, leading to a smaller population of positive cells. Such a technique was used in the thesis to analyze all of the flow cytometry results. Some researchers consider cells exhibiting more than a set value of fluorescent than the control sample after standardizing the control sample's normal distribution at a set fluorescent value, regardless of the distribution width, as positive. This leads to an interpretation of more positive cells than the technique used in this thesis. Many articles do not describe in detail on how they analyzed their flow cytometry results. Due to many different possibilities for flow cytometry interpretations, it is difficult to compare the transfection rates between literatures.

Furthermore, flow cytometry is a very sensitive technique where miniscule changes may lead to a notable difference. For example, there will be difference for cells analyzed when it is freshly harvested compared to cells analyzed after they were fixed in paraformaldehyde. This

difference arises from the fluorescent signals of paraformaldehyde [102]. Flow cytometry is a powerful instrument with high sensitivity. However, many caution and clarification is required to standardize the use of the machine.

5.3.4 - Confocal Microscope

The thesis could have been stronger with the visual support from confocal microscope. However, confocal microscope is less sensitive and will need strong or fluorescent signal from the cells to show up on images. There will only be very few cells, if any, which will express fluorescent level detectable by confocal microscope. A much higher concentration of sonoporation marker will be needed for cells to express at the level required for confocal detection. There is a complication with flow cytometry if the fluorescent intensity was to be increased. It is possible that higher intensity will not be registered in flow cytometry, as the machine is only capable of registering fluorescent intensity 1000-fold of the background fluorescent level radiating from the control group. Any cells with higher fluorescent intensities will be recorded as the max intensity, hence losing information. It is necessary to have two experimental conditions: one for flow cytometry analyses, one for confocal microscope analyses. The two conditions with different concentration of sonoporation markers will not be describing the same experiment. Hence, it is not possible to incorporate confocal microscope results into the studies when flow cytometry is used to quantitatively describe the results.

5.3.5 - Electron Microscope and Multisizer

Currently the self-developed phospholipid microbubbles are viewed and characterized with an optical microscope. These optical microscope are capable of 400x magnification, but is not enough to precisely measure the sizes of the bubbles. Other instrumentations can help

better characterize those microbubbles. The use of electron microscope and multisizer can help with characterization.

Scanning Electron Microscope (SEM) can help look at the surface of the microbubbles, which will help to determining if a bubble was formed and if they were bursted after sonication. Transmission Electron Microscope (TEM) can help define the shape and sizes of bubbles. The electron microscope technique will gather information from a few samples, while the multisizer can measure the size of all the bubbles in solution.

These three instrumentation are available at the University of Alberta, but were not studied with these instruments due to training requirements and lack of responses from facilities assistants. However, as development of these microbubbles advances and ready for literature publication, it is necessary to incorporate these highly specific instruments in physical characterization of the microbubbles.

5.3.6 - Cell Cycle Analysis

As mentioned previously, it was believed that the variability of transfection rate and cell viability in sonoporation is due to the varying percentage of cells at different cell cycle from batch to batch. To eliminate the variation in results, triplicate of samples should be treated together using the same batch of cells. This would, in theory, give rise to results that have the smallest variance. However, the problem of non-repeatability and credibility would still be present. Therefore, it is necessary to record and present the state of cells when they were sonoporated.

Unfortunately, it is difficult to measure the population's cell cycle state for cancer cell lines. A cell cycle analysis kit, DNA Nuclear-ID™ Green Cell Analysis Kit, was bought from Enzo Life Science. The cells were stains and treated with the cell cycle analysis kit before analyzed

under flow cytometry. A negative control was prepared by adding reagents to arrest the cell population at M-phase. After 24 hours of growth, all the cells should have been in M-phase. Cells were stained and compared against as the negative controls. However, the negative controls for KG-1 cells used in this thesis did not show up as a narrow peak, as it should on the flow cytometry results. The width of the peak was abnormally wide, leading to an unreliable control. Many methods were used to narrow the band by increasing the permeability of the cells and by increasing staining time. However, none of these methods led to a narrow band required for credible flow cytometry histogram. Therefore, a new chemical or protocol is needed to define the state of cells.

5.3.7 – Cell Culture

To best stabilize the cell culture, many factors in growing cells must be taken into account. However, there are many uncontrollable variations which are inherent and difficult to control. For example, growth medium are different between lots. There are slight differences in concentration of nutrients between every batch of medium. This is a normal variance in medium manufacturing and is not controllable. The cell age, or the number of passages the population had gone through also poses problems in keep the cell culture the same from experiment to experiment.

Many other human related factors cause variations in growth conditions and are difficult to remove. All equipments in the lab are shared among many students, lab technicians and post-doctoral fellow. This sharing of equipment will lead to a variation in growing condition because every time the incubation door opens, CO_2 and temperature fluctuates. The frequency and duration of opening the incubator door can vary from day to day. This could drastically alter the growing conditions. Furthermore, it is also possible that an incubator contamination occurs; it

could be caused by one careless step from one user, resulting in an incubator-wide contamination. Another extra week would be required to re-culture all of the cells again and prepare for experiments. A single culture contamination can occur with an unclean hood and experimental equipment. Keeping the lab contamination free is a collaborative effort and is difficult to prevent accidents from happening.

Harvesting and splitting cells at a regular basis would help to keep cells more consistent from experiment to experiment. However, the growth rate may differ, leading to harvesting and splitting at a less than optimal timing. Moreover, since fume hoods and equipment are shared, hood might be occupied by other lab mates due to their experiment. There are difficulties logistically when trying to keep everything exactly constant from batch to batch.

There were also incidents where incubator were not looked after and would have caused cells to grow differently. For example, CO_2 tank may go empty, without previous notification, in the middle of the night or during weekend, impeding the growth rate and possibly the physiology of cells. An inadequate amount of water might cause the incubator's humidity to change or cause the medium to evaporate, which negatively affect the cell cultures. A constant monitor and maintenance of the incubator is also necessary to keep cell cultures growing optimally.

Reference

1- Cerchia, L., Franciscis, V. "Targeting cancer cells with nucleic acid aptamers." *Trends in Biotechnology (*2010), vol. 28, no. 10, pp. 517–525.

2 - Osman, R., Beyer, T. "Whole-body hybrid PET/MRI: ready for clinical use?" *European Journal of Nuclear Medicine and Molecular Imaging* (April 5 2011), vol. 38:992–995.

3- Song, K., *et al*. "Systematic study of enhanced cytotoxicity effects of gold-based nanoparticles in targeted cancer radiotherapy," 2009 IEEE/NIH Life Science Systems and Applications Workshop, LiSSA 2009, Article number 4906702, Pages 33-35.

4 - Alvarez-Salas, L.M. "Nucleic Acids as Therapeutic Agents." *Current Topics in Medicinal Chemistry (*2008*),* vol. 8, no.15, pp. 1379-1404.

5 - Lodish, Harvey F. Molecular cell biology. 6th Ed. New York: W.H. Freeman, 2008. Print

6 - Alberts, Bruce. Molecular Biology of The Cell. 4th Ed. New York: Garland Science, 2002. Print.

7 - Check, E. "Second cancer case halts gene-therapy trials." *Nature* 421 (2003), p. 305.

8 - Ilarduya, C.T., Sun, Y., Duzgunes, N. "Gene delivery by lipoplexes and polyp lexes." *European Journal of Pharmaceutical Sciences* (2010), 40, 159–170.

9 - Tarahovsky, Y. S. "Cell Transfection by DNALipid Complexes – Lipoplexes." *Biochemistry*(Moscow) (2009), vol. 74, no. 12, pp. 1293- 1304.

10 - Hagstrom, J.E., Ludtke, J.J., Bassik, M.C., Sebestyen, M.G., Adam, S.A., Wolff, J.A. "Nuclear import of DNA in digitonin-permeabilized cells." *Journal of Cell Sciences* (1997), 110, 2323–2331.

11 - Montier, T., Benvegnu, T., Jaffrès, PA., Yaouanc, JJ., Lehn, P. "Progress in Cationic Lipid-Mediated Gene Transfection: A Series of Bio-Inspired Lipids as an Example." *Current Gene Therapy (*2008), vol. 8, no. 5, pp. 296-312.

12 - Royal Society. 2004. Nanoscience and nanotechnologies: opportunities and uncertainties, RS Policy Document 19/04.

13 - McBain, S.C., Yiu, H.H.P., Dobson, J. "Magnetic nanoparticles for gene and drug delivery." *International Journal of Nan medicine (*2008), 3(2): 169-180.

14 - Cho, K., Wang, X., Nie, S., Chen, Z. (Georgia), Shin, D.M. "Therapeutic Nanoparticles for Drug Delivery in Cancer." *Clinical Cancer Research (*March 3 2008), 14:1310-1316.

15 - Niidome, T., Huang, L . "Gene Therapy Progress and Prospects: Nonviral Vectors." *Gene Therapy* (2002), 9: 1647–1652.

16 - Lin, M.T.S., Pulkkinen, L., Uitto, J., Yoon, K. " The gene gun: current applications in cutaneous gene therapy." *International Journal of Dermatology (*2000), 39: 161-170.

17 - Kuriyama, S., *et al*. "Particle-mediated gene transfer into murine livers using a newly developed gene gun." *Gene Therapy (*2000), 7: 1132–1136.

18 - Tsong, T.Y. "Electroporation of cell membranes." *Biophysical Journal (*August 1991)*,* vol. 60, pp. 297-306.

19 - Somiari, S., Glasspool-Malone, J., Drabick, J.J., Gilbert, R.A., Heller, R., Jaroszeski, M.J., Malone, R.W. "Theory and *in Vivo* Application of Electroporative Gene Delivery." *Molecular Therapy (*Sept. 2000)*,* vol. 2, no. 3, pp 178- 187.

20 - Dunny, G.M., Lee, L.N., LeBlanc, D. J. "Improved Electroporation and Cloning Vector System for Gram-Positive Bacteria." *Applied and Environmental Microbiology (*Apr. 1991), vol. 57, no. 4, pp. 1194-1201.

21 - Wells, D.J. "Gene Therapy Progress and Prospects: Electroporation and other physical methods." *Gene Therapy* (August 5 2004), 11: 1363–1369.

22 - Gehl, J. "Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research."*Acta Physiol Scand* (2003), 177: 437–447.

23 - Gao, X., Kim, KS., Liu, D. "Nonviral Gene Delivery: What We Know and What Is Next" *The AAPS Journal (*March 23 2007), 9(1), Article 9 (http://www.aapsj.org).

24 - Li, Y.S., Davidson, E., Reid, C.N., McHale, A.P. "Optimising ultrasound-mediated gene transfer (sonoporation) in vitro and prolonged expression of a transgene in vivo: Potential applications for gene therapy of cancer." *Cancer Letters* (2009), 273: 62–69.

25 - Miller, D.L., Pislaru, S.V., Greenleaf, J.F. "Sonoporation: Mechanical DNA Delivery by Ultrasonic Cavitation." *Somatic Cell and Molecular Genetics* (November 2002)*,* vol. 27, nos.1/6, pp. 115- 134.

26 - Huber, P.E., Pfisterer, P. "In vitro and in vivo transfection of plasmid DNA in the Dunning prostate tumor R3327-AT1 is enhanced by focused ultrasound." *Gene Therapy* (2000), 7: 1516-1525.

27 - Kim, HJ., *et al*. "Ultrasound-mediated transfection of mammalian cells." *Hum Gene Therapy (*1996), 7: 1339-1346.

28 - Tsunoda, S., *et al*. "Sonoporation using microbubble BR14 promotes pDNA/siRNA transduction to murine heart." *Biochemical and Biophysical Research Communications* (2005), 336: 118–127.

29 - Duncan, George. Physics for Biologist. New York. John Wiley & Sons,1975. Print.

30 - Hudson, Alvin. University Physics. 2nd Ed. New York. Saunders College Publishing. 1990. Print.

31 - Halliday, David. Fundamentals of Physics, 3rd Ed. New York. John Wiley & Sons,1988. Print.

32- Bueche, Frederick. Principles of Physics, 6th Ed. New York. McGraw-Hill, Inc, 2005. Print.

33 - Davidovist, Paul. Pysics in Biology and Medicine. 3rd Ed. London. Academic Press, 2008. Print.

34 - Hofbauer, J., Hobarth, K., Marberger, M. "Lithoclast: New and Inexpensive Mode of Intracorporeal Lithotripsy." *Journal of End urology (*December 1992), 6(6): 429-432.

35- Haar, G.T. "Ultrasound Focal Beam Surgery." *Ultrasound in Med Biol* (1995), 21(9):1089-1100

36 - Haar, G.T. "Therapeutic applications of ultrasound." *Progress in Biophysics and Molecular Biology* (2007), 93: 111–129.

37 - Woon Tiong Ang; Scurtescu, C.; Wing Hoy; El-Bialy, T.; Ying Yin Tsui; Jie Chen; , "Design and Implementation of Therapeutic Ultrasound Generating Circuit for Dental Tissue Formation and Tooth-Root Healing,"*Biomedical Circuits and Systems, IEEE Transactions on* , vol.4, no.1, pp.49-61, Feb. 2010

38 - Von Ramm, O.T., Smith, S.W. "Real Time Volumetric Ultrasound lmaging System." *Journal of Digital Imaging (*November1990)*,* vol. 3, no. 4, pp 261-266.

39 - Tranquart, F., Grenier, N., Eder, V., Pourcelot, L. " Clinical use of Ultrasound Tissue Harmonic Imaging."*Ultrasound in Medicine & Biology (*1999)*,* vol. 25, no. 6, pp. 889–894.

40 - Burns, P., Wilson, S. "Microbubbles Contrast for Radiological Imaging: 1. Principles," *Ultrasound Quarterly* (March 2006), vol. 22, no.1

41 - "Vscan". *GE Healthcare.* 1Aug. 2011. Web. <http://www.gehealthcare.com/euen/ultrasound/products/portable/vscan/index.html .>

42 - Helguera, M. "An introduction to Ultrasound." *Chester F. Carlson Center for Imaging Science, Biomedical and Materials Multimodal Imaging Laboratory, Rochester Institute of Technology.* 1 Aug. 2011. Web. <http://www.cis.rit.edu/research/biomedical/ultrasoundintro/ultraintro.html>

43 - Li, Carina. "Basic Physics of Ultrasound." Department of Anaesthesia and Intensive Care, The Chinese University of Hong Kong. 1 Aug. 2011. Web. <http://www.usgraweb.hk/en/Pdf%20Slide%20Show/Basic%20Physics%20of%20Ultrasound.pdf>

44 - Perry Sprawls, The Physical Principles of Medical Imaging, 2nd Ed. Chicago. Wolters Kluwer Law & Business. 1993. Print.

45 - Hendee, William R. *Medical Imaging Physics*, Fourth Edition, New York, Wiley-Liss.2002. Pirnt.

46 - Lawrie, A. *et al.*"Microbubble-enchanced Ultrasound for Vascular Gene Delivery," Gene Therapy (2000), 7:2023-2027

47 - Gramiak, R., MD, Shah, P.M., MD. "Echocardiography of the Aortic Root." *Investigative Radiology (*1968), vol. 3, no.5, pp. 356-366.

48 - Frinking ,P.J.A., Bouake, A., Kirkhorn, J., Ten Cate, F.J., De Jong, N. "Ultrasound Contrast Imaging: Current and New Potential Methods." *Ultrasound in Med. & Biol. (*2000)*,* vol. 26, no. 6, pp. 965–975.

49 - Ophir, J., Parker, K.J. "Contrast agents in diagnostic ultrasound." *Ultrasound Med Biol (*1989), 15(4): 319 –33.

50 - Caiati, C., *et al*. "New Noninvasive Method for Coronary Flow Reserve Assessment: Contrast-Enhanced Transthoracic Second Harmonic Echo Doppler." *Circulation* (1999), vol. 99, iss. 6, pp. 771-778.

51 - De Jong, N., Bouakaz, A., Frinking, P. "Basic Acoustic Properties of Microbubbles." *Echocardiography (*April 2002), vol. 19, no. 3, pp. 229- 240.

52 - Blomley, M.J.K., Cooke, J.C., Unger, E.C., Monaghan, M.J., Cosgrove, D.O. "Microbubble contrast agents: a new era in ultrasound." *BMJ (*2001), 322: 1222- 1225.

53 - Harvey, C. J., Albrecht, T. "Ultrasound of focal liver lesions." *Eur. Radiology* (2001), 11: 1578-1593.

54 - Correas, JM., Bridal, L., Lesavre, A., Møjean, A., Claudon, M., HØlØnon, O. "Ultrasound contrast agents: properties, principles of action, tolerance, and artifacts." *Eur. Radiol.* (2001), 11: 1316-1328.

55 - Quaia E (2005) Classification and safety of microbubble-based contrast agents. In: Quaia E (ed) Contrast media in ultrasonography: Basic principles and clinical applications. Springer, Berlin Heidelberg New York, pp 43–70

56 - Cosgrove, David. "Ultrasound contrast agents: An overview." *European Journal of Radiology* (2006), 60: 324–330.

57 - Quaia, E. "Microbubble ultrasound contrast agents: an update." *Eur. Radiology* (2007), 17: 1995–2008.

58 - Klibanov, A.L. "Targeted delivery of gas-filled microspheres, contrast agents for ultrasound imaging." *Advanced Drug Delivery Reviews* (1999), 37: 139–157.

59 - Hernot, S., Klibanov, A.L. “Microbubbles in ultrasound-triggered drug and gene delivery.” *Advanced Drug Delivery Reviews* (2008), 60: 1153- 1166.

60 - De Jong, N., *et al*. “Principles and recent developments in ultrasound contrast agents.” *Ultrasonics* (July 1991), vol. 29, pp. 324- 330.

61 - Narayan, P., Wheatley, M.A. "Preparation and Characterization of Hollow Microcapsules for Use as Ultrasound Contrast Agents," *Polymer Engineering and Science* (Novemeber 19999), vol. 39, no. 11:2242-2255

62 - Cheung, J.S., Chow, A.M., Guo, H., Wu, E.X. “Microbubbles as a novel contrast agent for brain MRI.” *NeuroImage* (2009), 46: 658–664.

63 - Pislaru, S.V., Pislaru, C., Kinnick, R.R., Singh, R., Gulati, R., Greenleaf ,J.F., Simari, R.D. “Optimization of ultrasound-mediated gene transfer: comparison of contrast agents and ultrasound modalities.” *European Heart Journal* (2003), 24: 1690–1698.

64 - Newman, C.M.H., Bettinger, T. “Gene therapy progress and prospects: Ultrasound for gene transfer.” *Gene Therapy* (2007), 14: 465- 475.

65 - Hallow, D.M., Mahajan, A.D., McCutchen, T.E., Prausnitz, M.R. “Measurement and correlation of acoustic cavitation with cellular bioeffects.” *Ultrasound Med Biol (*2006), vol 32, no.7, pp. 1111–1122.

66 - Zhou, Y., Cui, J., Deng, C.X. “Dynamics of Sonoporation Correlated with Acoustic Cavitation Activities.” *Biophysical Journal (*April 2008*),* vol. 94, iss. 7, pp. L51- L53.

67 - Ward, M., Wu, J., Chiu, JF. “Ultrasound-induced cell lysis and sonoporation enhanced by contrast agents.” *J. Acoust. Soc. Am. (*May 1999*),* vol. 105, no. 5, pp. 2951- 2957.

68 - Shi, W., *et al.* "Destruction of Contrast Microbubbles and the Association With Inertial Cavitation," Ultrasound in Med Biol (2000), vol 26, no. 6, pp. 1009-1019.

69 - Miller, D.L., Thomas, R.M. “Ultrasound Contrast Agents Nucleate Inertial Cavitation in Vitro.” *Ultrasound in Med. & Biol.* (1995*),* vol. 21, no. 8, pp. 1059- 1065.

70 - National Council on Radiation Protection and Measurements (NCRP) Exposure criteria for medical diagnostic ultrasound: I. Criteria based on thermal mechanisms. NCRP Report No. 113. Bethesda, MD: NCRP, 1992.

71 - Miller, D.L., Thomas, R.M., Frazier, M.E. “Ultrasonic Cavitation Indirectly Induces Single Strand Breaks in DNA of Viable Cells in Vitro by The Action of Residual Hydrogen Peroxide.” *Ultrasound in Med & Biol* (1991), 171: 729-735.

72 - Church, C.C. “A theoretical study of cavitation generated by an extracorporeal shock wave lithotripter.” *J Acoust Sot Am (*1989), vol. 86, iss. 1, pp. 215-227.

73 - Miller, D.L. “A review of the ultrasonic bioeffectiveness of microsonation, gas-body activation, and related cavitation-like phenomena.” *Ultrasound Med Biol (*1987), 13: 443-470.

74 - Gormley, G., Wu, J. “Observation of acoustic streaming near Albunex® spheres.” *J. Acoust. Soc. Am*. (November 1998), 104(5): 3115-3118.

75 - Forbes, M.M., Steinberg, R.L., O’Brien, W.D., Jr. “Examination of Internal Cavitation of Optison in Producing Sonoporation of Chinese Hamster Ovary Cells.” *Ultrasound in Med. & Biol*. (2008), vol. 34, no. 12, pp. 2009–2018.

76 - Pan, G. J., *et al* ."Stem Cell Pluripotency and Transcription Factor Oct4," *Cell Research* (2002), 12(5-6):321-329

77 - Reya, T., *et al*. "Stem Cells, Cancer, and Cancer Stem Cells," *Nature* (November 2001) 414:105-111

78 - Vats, A., Bielby, R.C., Tolley, N.S., Nerem, R., Polak, J.M. “Stem cells.” *Lancet (*Aug 13-19 2005), 366 (9485): 592-602.

79 - Weissman, I.L. “Stem Cells: Units of Development, Units of Regeneration, and Units in Evolution.” *Cell (*Jan 7 2000), 100(1): 157-168.

80 - Hwang, W.S., *et al.*“Evidence of a Pluripotent Human Embryonic Stem Cell Line Derived from a Cloned Blastocyst.” *Science (*March 12 2004), vol. 303, pp. 1669- 1673.

81 - Odorico, J.S., Kaufman, D.S., Thomson, J.A. “Multilineage Differentiation from Human Embryonic Stem Cell Lines.” *Stem Cells* (2001), 19:193-204.

82 - Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M. “Embryonic Stem Cell Lines Derived from Human Blastocysts.” *Science* (November 6 1998), vol. 282, pp. 1145- 1147.

83 - Dennis, J.E., Carbillet, J.P., Caplan, A.I., Charbord, P. “The STRO1+ marrow cell population is multipotential.” *Cells Tissues Organs* (2002), 170: 73-82.

84 - Toma, J.G., Akhavan, M., Fernandes, K.J.L., Barnabe-Heider, F., Sadikot, A., Kaplan, D.R., Miller, F.D. “Isolation of multipotent adult stem cells from the dermis of mammalian skin.” *Nature Cell Biology* (2001), vol. 3, pp. 778-784.

85 - Scholer HR. "The Potential of Stem Cells: An Inventory. Human Biotechnology as Social Challenge," Ashgate Publishing, Ltd. 2007; 28-3

86 - Weissman, I.L., Anderson, D.J., Gage, F. “Stem and Progenitor Cells: Origins, Phenotypes, Lineage Commitments, and Transdifferentiations.” *Annual Review of Cell and Developmental Biology* (2001), 17: 387- 403.

87 - Muller-Sieburg, C.E., Cho, R.H., Thoman, M., Adkins, B., Sieburg, H.B. “Deterministic regulation of hematopoietic stem cell self-renewal and differentiation.” *Blood* (2002), 100: 1302-1309

88 - Ema, H., Kobayashi, T., Nakauchi, H. "Principles of Hematopoietic Stem Cell Biology," Hematopoiet Stem Cell Biology. Ed Montonari Kondo. Humana Press, 2010. Print.

89 - Gul, H., *et al*. “Valproic acid increases CXCR4 expression in hematopoietic stem/ progenitor cells by chromatin remodeling.” *Stem Cell and Development* (2009), 18 (6): 831-838.

90 - Ciccarone. V., *et al*. “Novel and optimized cationic lipid reagents for DNA and RNA transfection of adherent and suspension mammalian and insect cells.“ *FASEB Journal* (1996), 10(6): A1529.

91 - Karshafian, R., Samac, S., Bevan, P., Burns, P.; "Microbubbles Mediated Sonoporation of Cells in Suspension: Clonogenic Viability and Influence of Molecular Size on Uptake," *Ultrasonics* (2010), 50:691-697.

92 - "MTT Assay". *Wikipedia.* 21Aug. 2011. Web. <http://en.wikipedia.org/wiki/MTT_assay>

93 – Carmichael , J., *et al.*; "Evaluation of a Tetrazolium-based Semiautomated Clorimetric Assay: Assessment of Chemosensitivity Testing," *Cancer Research* (1987); 47:936-942.

94 - Bao, S., Thrall, B., and Miller, D.; "Transfection of A Reporter Plasmid Into Cultured Cells By Sonoporation In Vitro," Ultrasound in Medicine & Biology (1997), 23(6):953-959.

95 - Hallow, D., Mahajan, A., McCutchen, T., Prausnitz, M.; "Measurement and Correlation of Acoustic Cavitaiton With Cellular Bioeffects," *Ultrasound in Medicine & Biology* (2006), 32(7): 1111-1122.

96 - Forbes, M., Steinberg, R., O'Brien, W.; "Frequency-Dependent Evaluation of hte Role of Definity in Producing Sonoporation of Chinese Hamster Ovary Cells," *Journal of Ultrasound Medicine* (2011), 30:61-69.

97 - Deshpande, M., Prausnitz, M.; " Synergistic Effect of Ultrasound and PEI on DNA transfeciton in vitro," *Journal of Controlled Release* (2007), 118:126-135.

98 - Reslan L., Mesta JL., *et al*.; “Transfection of cells in suspension by ultrasound cavitation," *Journal of Controlled Release* (2010), 142: 251-258.

99 – Kudo, N., Okada, K., Yamamoto, K.; "Sonoporation by Single-Shot Pulsed Ultrasound with Microbubbles Adjacent to Cells," *Biophysical Journal* (2009), 96:4866-4876

100 - Zhou, Y., Kumon, R., Cui, K., Deng, C.; "The Size of Sonopration Pores On The Cell Membrane," *Ultarsound in Medicine & Biology* (2009), 35(10):1756-1760

101 – Le Gac, S., Zwaan, E., Van Der Berg, A., Ohl, C.; "Sonopration Ff Suspension Cells With A Single Cavitation Bubble In A Microfluidic Confinement," *Lab Chip* (2007), 7:1666-1672

102 - Watson, James. Flow Cytometry Data Analysis: Basic Concepts and Statistics. Cambridge: Cambridge University Press. 2005. Print

103 - Leighton, T.; "The Acoustic "Gene therapy progress and prospects: Ultrasound for gene transfer Revised and Expanded", Marcel Dekker, Inc., pp4

104 - Patrick, B., Valerie, P.C., Adolfo, G., *et al.*; "Naked DNA Injection for liver metastases treatment in rats," *Hepatology* (2002), 35:1144–1152.

105 - Schlicher, R., *et al.;* "Changes in Cell Morphology Due to Plasma Membrane Wounding By Acoustic Cavitation," *Ultrasound in Medicine & Biology* (2010), 36(4):677-692

106 - Cheon, S.H., *et al,;* "Enhanced Delivery of siRNA Complexes By Sonoporation In Transgeneic Rice Cell Suspension Cultures," *Journal of Microbiology Biotechnology* (2009), 19(8):781-786

107 - Greco, A., *et al,;* "Eradication of Therapy-Resistant Huiman Prostate Tumors Using an Ultrasound-Guided Site-Specific Cancer Terminator Virus Delivery Approach," *Molecular Therapy* (2010), 18(2):295-306

108 - Casey, G., *et al,;* "Sonoporation Mediated Immunogene Therapy of Solid Tumors," *Ultrasound in Medicine & Biology* (2010), 30(3):430-440

109 - Blanco, E., *et al,;* "Nanomedicine In Cancer Therapy: Innovative Trends And Prospects," *Cancer Science* (2011), 102(7):1247-1252

110 - Escoffre, J.M., Kaddur, K., Rols, M.P., Bouakaz, A.; "In Vitro Gene Transfer By Electrosonoporation," *Ultraound in Medicine & Biology* (2010), 36(10):1746-1755

111 - Escoffre, J.M., Piron, J., Novell, A., Bouakaz, A.; "Doxorubicin Delivery Into Tumor Cells With Ultrasound and Microbubbles," *Mol. Pharmaceutics* (2011), *8*(3):799–806

112 - Husseini, G., Pitt, W.; "Micelles And Nanoparticles For Ultrasonic Drug and Gene Delivery," *Advance Drug Delivery Reviews* (2008), 60:1137-1152

113 - Mehier-Humbert, S., Bettinger, T., Yan, F., Guy, R.; "Influence Of Polymer Adjuvants on the Ultrasound-Mediated Transfection of Cells In Culture," *Europeian Journal of Pharmaceutics and Biopharaceutics* (2009), 72:567-573

114 - Morrison, S., Shah, N., Anderson, D.; "Regulatory Mechanism in Stem Cell Biology," *Cell* (1997), 88:287-298

115 - Sboros, V.; "Response Of Contrast Agents To Ultrasound," *Advance Drug Delivery Reviews* (2008), 60:1117-1136

116 - Weissman, I.; "Stem Cells: Units Of Development, Units Of Regeneration, and Units In Evolution," *Cells* (2000), 100:157-168

Publication

Wong, P., Choi, M.A., Gul-Uludag, H., Ang, W.T., Xu, P., Xing, J., Chen, J.; "Ultrasound-mediated gene delivery into hard-to-transfect KG-1 cells," *Proceedings of the 2011 IEEE/NIH Life Science Systems and Applications Workshop* (2011), P.143-146.