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## **Destruction of Moving Leukemia Cancer Cells In an Extra-Corporeal system using Continuous Low-frequency Ultrasound**

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## ABSTRACT

Low frequency ultrasound has been used for therapeutic purposes since a long time. It is non-invasive and thus application for medical purpose is easier. This method has been used in labs for preclinical in-vitro settings to observe the effects of low frequency ultrasound sound on neoplastic cells lines. The human leukemia cell lines are sonic sensitive and when exposed to various low frequencies of ultrasound for an amount of time, can cause their lysis and decrease their viability. The project focuses on using low frequency ultrasound on syringe pump mediated flowing leukemia cell lines and see how much their sonic sensitivity varies when they are in motion in comparison to a control and when they are stationary. Tygon tubes are used to carry out the experiments and low frequency ultrasound is mediated by using a 20khz system at 30% amplitude. The cells were exposed to continuous sonication for varying time durations and varying flow rates and the end effects were noted. Various tests were performed to show the preferential damage of leukemia cell lines like apoptosis assays, actin staining and trypan blue exclusion. Based on the results obtained it showed that the sonic sensitivity is affected by motion and there is more damage to the neoplastic cells in comparison to when they are stationary sonicated. In future these studies will help to formulate a setup to analyze how much the sonic sensitivity will vary when the leukemia cells are mixed with whole blood cells and exposed to moving sonications. These studies may create a base for in-vivo experiments, as the results will give us an understanding about the exposure time which will cause maximum damage to the cells and how much time should the mouse be exposed to sonication to get preferential damage.

# **Destruction of Moving Leukemia Cancer Cells in an Extra-corporeal System using Continuous Low-Frequency Ultrasound**

By:

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B.S Biotechnology Engineering, Mumbai University, 2016

Thesis

Submitted in partial fulfillment of the requirements for the degree of  
Master of Science in Bioengineering

Syracuse University

June 2018

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## 1) INTRODUCTION

### 1.1 What is Leukemia?

Leukemia is a neoplasm that is inherently systemic and malignant because it involves blood cells and their stem cells and appears in the circulation. Leukemias account for 3.7% of all new cancer cases. There are over 60,000 new cases every year and cause around 24,000 deaths in US. It is a malignancy of normal blood cells and causes the abnormal growth and differentiation of blood cells in the bone marrow<sup>[n]</sup>. These abnormal cells are usually white blood cells, but they don't function normally eventually causing the disruption of the normal functioning of the blood cells which includes fighting infection, transporting oxygen and controlling bleeding<sup>[n]</sup>. Leukemia usually starts by the disruption of the blood forming cells (Figure 1). The blood cells are formed through hematopoiesis which involves the hematopoietic stem cells. Hematopoiesis takes place in the bone marrow and occurs by the division of the stem cells into various cell lineages<sup>[a]</sup>. The HSCs (Hematopoietic stem cells) are self-renewing cells and thus one of the daughter cells always remain as an HSC after division. The HSCs divide into two lineages, Myeloid or Lymphoid progenitor cells which later give rise to all the blood cells (Figure 2). The myeloid precursor cells give rise to blood cell lineages that include erythrocytes, megakaryocytes, platelets, granulocytes. The lymphoid precursor cells give rise lymphocytes that includes B-cells, T-cells and natural killer cells<sup>[o]</sup>.



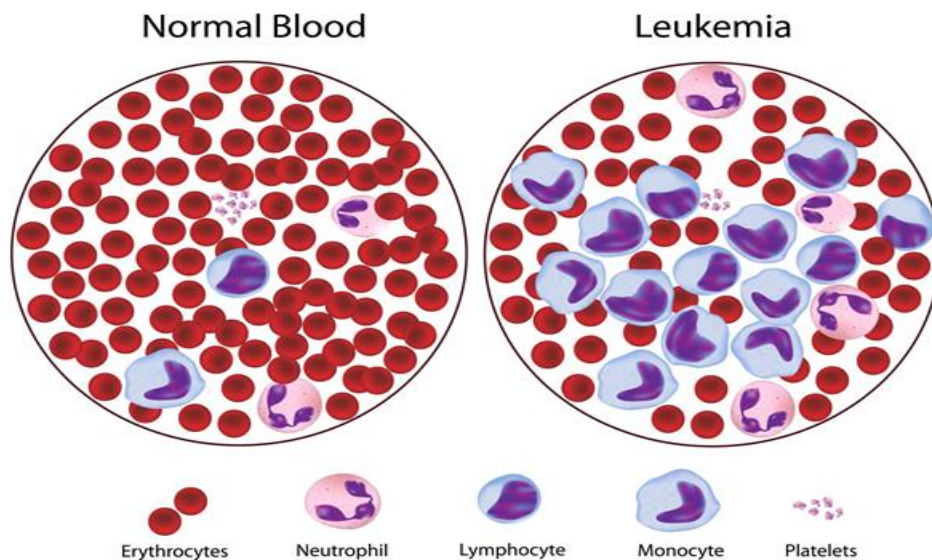


Figure 1: Normal vs leukemia cells [i]

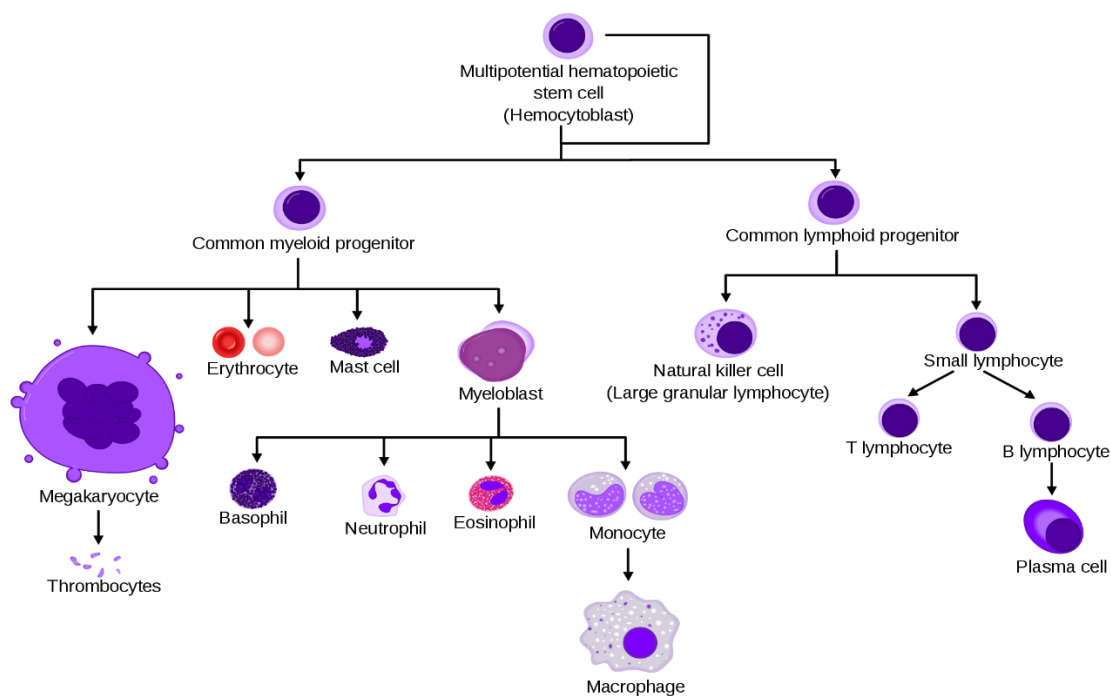


Figure 2: Diagram showing development of different blood cells from hematopoietic stem cells to mature cells [r]

During the onset of leukemia, there is an abnormal division of the myeloid and lymphoid progenitor cells rising from mutations in the DNA of the cells which causes them to lose their normal functions<sup>[o]</sup>. Mutations are usually caused by various genetic and environmental factors like chromosome translocation, genetic abnormalities like Down's syndrome, exposure to certain chemicals such as benzene, smoking or a familial history of leukemia<sup>[a]</sup>. Leukemia has been difficult to diagnose at its early stages and thus it becomes difficult to prevent it, it is usually diagnosed in its later stages and it tends to spread rapidly through the blood stream<sup>[n]</sup>. The leukemia cells are either acute which are immature, or they are chronic which are much more like the normal white blood cells. Based on the type of blood cells effected, leukemia is divided Acute or Chronic.

### **1.2 Acute Lymphocytic Leukemia (ALL)**

It's a type of cancer which starts on the early stages of white cells called lymphocytes in the bone marrow. This type of leukemia is not that common in adults, but maximum cases are diagnosed in children in the age range of two to five. In US it is the most common cancer-causing death in children. This type of cancer shows symptoms like weakness, anemia, dizziness, headaches, breathlessness, enlarged lymph nodes, loss of weight, unexplained excessive bruises. This type of leukemia is caused by various genetic abnormalities and generally occurs during conception, inheriting some of the mutated genes. <sup>[a][n]</sup>

### **1.3 Acute Myeloid Leukemia (AML)**

It is a type of leukemia which causes the production of abnormal myeloblasts, red blood cells and platelets in the bone marrow. This type of leukemia is common in adults and may worsen if not treated quickly. The production of abnormal cells can keep on increasing with time and can

decrease the place for normal blood cells in the blood and bone marrow leading to conditions of easy bleeding, anemia, infections and it can spread to different parts of the body including the skin, gums and central nervous system<sup>[n]</sup>. It is usually caused due to smoking, radiation and in case the person has been treated previously with chemotherapy. It has subtypes depending on the stage of maturity of the neoplastic cells. Acute promyelocytic leukemia is a subtype of AML, which causes bleeding and blood clots and it occurs when two parts of genes stick together (chromosomal translocation).<sup>[a][b]</sup>

#### **1.4 Chronic Lymphocytic Leukemia**

It is the second most common type of leukemia in adults, but it is rarely found in children. In chronic cases, the body produces an abnormal number of lymphocytes which leads to less room for the healthy ones. This causes a decrease in immunity and the body is exposed to infections which it is unable to fight. This type of leukemia progresses slowly which makes it difficult to detect and thus it worsens the case. All this leads to various symptoms like enlarged lymph nodes, weight loss, fever and infection, pain in the ribs. It usually occurs after the age of 50 and males are more prone to this disease than females.<sup>[a][n]</sup>

#### **1.5 Chronic Myeloid Leukemia**

It is a type of clonal bone marrow stem cell disorder which is caused when section of two different chromosomes switch places and make a new abnormal one leading to the formation of abnormal myeloid cells in the bone marrow and accumulating in the blood stream. It is like CLL in terms of the age group that is people over 60 years of age are diagnosed with this disease and more males are prone to it than females. It is the first type of cancer which is directly linked to genetic abnormality that is chromosomal translocation. It usually is classified into three stages

based on its characteristics<sup>[n]</sup>. First is the chronic phase which is the initial stage of the disease where maximum people are diagnosed. After several years it moves from chronic to an accelerated phase where the number of different abnormal myeloid cells are more than the criteria put forward by WHO. The last phase is the blast crisis, where CML behaves like an acute leukemia where there is less chance of survival and it progresses quickly. It is diagnosed if the patient has more than 20% myeloblasts in the bone marrow and if there is a development of chloroma. <sup>[a][b]</sup>

## **1.6 Treatment of Leukemia**

Various treatments have been developed and used for treating leukemia. The aim of treatment is to destroy the abnormal leukemia cells and allow the normal healthy blood cells to form in the marrow. The basic goal is to diagnose leukemia at early stages and to treat the abnormalities through various ways such as chemotherapy, radiation therapy, stem cell therapy and biological therapy. Leukemia is diagnosed through blood counts, taking samples of bone marrow and immunophenotyping to look for genetic alterations and expression of various cell surface markers by the leukemia cells. <sup>[a]</sup> The treatment basically depends on the type of leukemia, age factor, health of the patient and whether the leukemia has spread to other body parts. Usually when a person has a chronic leukemia, there are no symptoms and doctors must do watchful waiting. Treatment is begun once the patient starts showing symptoms. The chronic cases are usually treated with chemotherapy with one or more drugs. Depending on the stage of the disease the doctor can go for a bone marrow transplant. Recently tyrosine kinase inhibitors have become the standard therapy for patients who are in the initial stages of the disease. There is also use of targeted therapies which has helped increase the survival time of many patients.

For acute leukemia where chemotherapy has been the treatment method, the patient goes through different stages: induction therapy, consolidation therapy and maintenance therapy. <sup>[a]</sup> Therapies also include radiation therapy and in many cases stem cell transplants. Recent treatment includes using immunotherapy where the patient's own immune system helps fight the disease. Use of various monoclonal antibodies has given results in treating ALL <sup>[k]</sup>. Doctors have started using combination therapy, that is to use two different therapies in combination to get a greater damage to these leukemia cells. Most of the methods used for treatment are invasive and all these come with certain side effects which in the long run creates some complications. <sup>[a]</sup>

## 1.7 Ultrasound Treatment

Ultrasound has been used since a long time in medical industry. It is used for imaging of various organs, assessment of vascularization and has been used largely due to its easy availability, low maintenance and high sensitivity. Apart from imaging, ultrasound has also been used for therapies and localized treatment of various diseases. High frequency ultrasound has been used in extracorporeal shockwave lithotripsy for treating kidney stones and gall bladder stones.

Ultrasound at different frequencies have different effects <sup>[d][e]</sup>. At high frequencies, there is production of both thermal and mechanical effects. At lower frequencies, there is production of cavitation bubbles which produce the rapid expansion and compression movement of waves from a sonotrode inside a liquid medium<sup>[c]</sup>. The cavitation occurs when the acoustic power inputs are high enough to cause the formation of microbubbles at a nucleation site within the liquid medium which tend to increase during the rarefying phase of a sound wave and later collapse during compression phase <sup>[c][p]</sup>. This collapse leads to powerful shock waves which pass through the liquid at a pressure as high as 300MPa. This is a conversion of sonic energy to mechanical

energy and the implosion of the microbubbles which creates localized conditions of high temperature and high pressure within the collapsed cavities causing cell damage and lysis<sup>[h]</sup>.

Overall cavitation is a process of microbubble nucleation, growth, and collapse caused by intense sonic waves.

Low frequency ultrasound has also been used for various medical applications. It tends to cause preferential damage in neoplastic cells, possibly based on the increased volume of neoplastic cells compared to normal cells, and thus has been used in cancer research. It has been seen that the neoplastic cells are sonic sensitive because of the induction of programmed cell death (apoptosis)<sup>[d][p]</sup>.

## **1.8 CELL SONIFIER**

Various cell sonifiers are used in laboratories for research on cell disruption. A cell sonifier is a versatile laboratory tool with the ability to produce sonication at a desired frequency and amplitude for cell disruption and various other biological and chemical processes. A Branson 20khz sonifier was used for experiments on the leukemia cell lines <sup>[d]</sup>. This system consists of a power source attached to a transducer and a sonotrode which produces the sonication. The system can generate power up to 200 watts and a frequency of 20khz. Various diameter horns can be connected for varying intensities and each intensity has a different effect on cell lysis and sonic sensitivity. The two main variables of the sonifier are the frequency and amplitude. Amplitude is the percent output that is the percentage of watts utilized by the system during sonication. For the experiments the amplitude was fixed at 30% because it produced the best results in terms of cavitation. At 20khz and 30% amplitude, larger cavitation bubbles were produced as compared to other frequencies and amplitude and thus was beneficial for the

experiments. The cavitation was validated and measured using a cavitation meter which show the intensity of cavitation in  $\text{W}/\text{cm}^2$ . [d]

### **1.9 Effect of cell movement on sonic sensitivity**

Experimental systems were designed to determine the effect of movement of sonic sensitivity of leukemia cells. In general, the blood flows at about 26-30ml/min in the arteries in our body. The average diameter of the arteries range between 800 microns and 1.8mm. A syringe pump was utilized for a synchronized to and fro movement of the cells inside a tygon tubing. Tygon is a polymer which is non-toxic and has been used in medical and pharmaceutical industries as well. It is one of the basic tubing for various pumping systems. The diameter of this tubing for the system was 3.175mm. The whole system consists of two syringe pumps linked to each other for the synchronized movement. To the syringe pumps, two syringes are attached respectively and the tygon tubing is attached to these syringes (See Figure 5). The configuration of the tubing is changed to check any varying effect. The tubing is placed either in direct contact with the sonotrode or at a distance from the sonotrode. This same system is used to expose the cells to sonication when they are stationary and when they are in motion and later compare the results. The main aim of this setup was to check whether there is more preferential damage of the cells when they are stationary and when they are moving. This result can help us know whether the movement of cells in vivo can be used as a target for preferential sonic damage.

This project aims on knowing how movement effects the lysing of cells through ultrasound and how does various parameters like flow rate, plane of sonication and time of exposure affect the results. Only 20khz system at 30% amplitude was used for the experiments and the cell lines used were U937 (Human Acute Monocytic Leukemia) and K562 (Human Chronic Myeloid Leukemia)

## **2) MATERIALS AND METHODS**

### **2.1 Preparation of Leukemia Cell lines**

Two leukemia cell lines were used, U937(Human acute monocytic leukemia, ATCC® CRL- 1593.2) and K562 (Human chronic myeloid leukemia, ATCC® CCL-243). These cells were placed at  $5.2 \times 10^4$  viable cells/ml in 20% fetal bovine serum (FBS) in Iscove's medium without glutamine, with the following added: 200 units/ml penicillin, 200 µg/ml streptomycin, 100 µg/ml gentamicin sulfate, 40 µM glutamine (50 µl of 2 mM glutamine per 5 ml medium), and 50 µl of amphotericin B (2.5 µg/ml concentration) per 5 ml of medium. Cells were incubated in 5% CO<sub>2</sub> in a humidified chamber at 37 °C. The cells were passaged after every 4-5 days by sub-culture to  $X \times 10^5$  cells/ml in new media. The cells were used for experiments when their concentration was in the range of  $3 \times 10^5$  to  $1 \times 10^6$  cells/ml.

### **2.2 Cell Viability**

The viability of the cells was assessed using trypan blue exclusion at 1:1 by volume. The trypan blue exclusion assay gives us the total concentration and viability of the cell lines. If the cells take up the trypan blue and are blue stained, they are non-viable cells. 10µl of the prepared cells are taken in an eppendorf tube and 10µl of 0.4% trypan blue stain is added to it and mixed well. This mixture is then taken on a cell counter slide and analyzed in a Countess II FL Automated cell counter. The cell counter detects the trypan blue stain and gives us the concentration, viability and the average size of all the cells in the mixture.



## 2.3 Ultrasound apparatus and intensity measurement

Sonication of cells was carried out using a 20 kHz Branson Sonifier (200watts). Two different sonotrode horns were used, one 19mm in diameter and another 60mm in diameter. 60mm diameter cup was used for both the sonotrodes. Deionized distilled water was degassed using a Vacuum and Buchner Flask. The cup is filled with this degassed water till 3cm above the top of the horn <sup>[d]</sup>. The system was set at an amplitude of 30% which gave the perfect wave and frequency of 20khz. The ultrasonic frequency was measured using an oscilloscope and hydrophone. The hydrophone is placed in a small vial placed on the sonotrode and the frequency was measured at various amplitudes. The oscilloscope showed the output frequency in the wave form. The sonifier was kept at continuous sonication to carry out the experiments. The cavitation intensity was measured using a cavitation meter. The cavitation meter was kept right above the sonotrode and the system was switched on and the intensity was calculated in terms of cavins where:

$$1 \text{ Cavin} = 0.0013 \text{ Watts/cm}^2$$

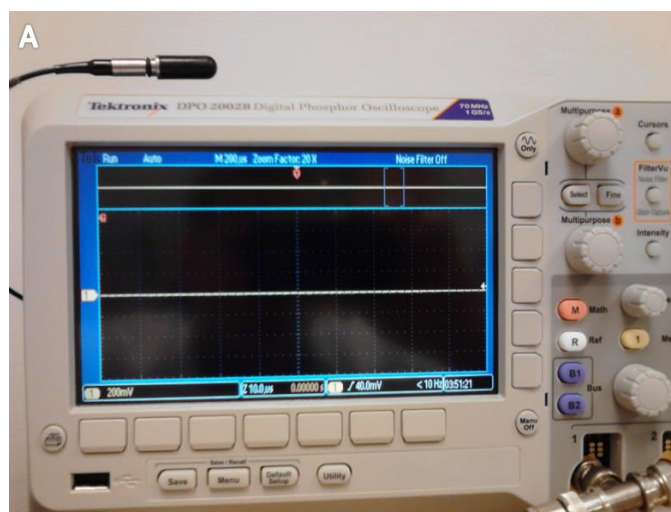


Figure 3: Validation of ultrasonic system by an oscilloscope and hydrophone to measure ultrasonic frequency (kHz). <sup>[d]</sup>



Figure 4: Validation of ultrasonic system's cavitation intensity by using a cavitation meter <sup>[d]</sup>

## 2.4 Extra-corporeal setup for moving cells

To access the sonic sensitivity of leukemia cells when they are in motion, a system was setup which used syringe pumps. Two syringe pumps were coupled with each other for a constant to and fro motion. The syringe pumps are connected with two 10ml syringes respectively. The pumps have a small digital screen which gives the value of the flow rate. The flow rate can be changed according to our experiments. When both the pumps are switched on, one pump withdraws the fluid and the other one pumps it and vice-versa. The syringes are connected to a tygon tubing in which the cells are present. The tygon tubing is placed above the sonotrode to expose the cells to sonication when they are stationary and when they are moving and then analyze their sonic sensitivity. The cells were even analyzed when they are in motion and not sonicated. This experiment helped us understand whether movement alone causes any damage to the cells. When stationary sonication is to be accessed, the tubing is just placed above the sonotrode without switching on the pumping and they are sonicated whereas in case of moving sonication, the pumps are switched on and the cells are sonicated as they are moving. The tubing is placed either planar or perpendicular to the field of sonication. Parameter such as time of exposure and flow rate are accessed to see the difference in sonic sensitivity based on these parameters.

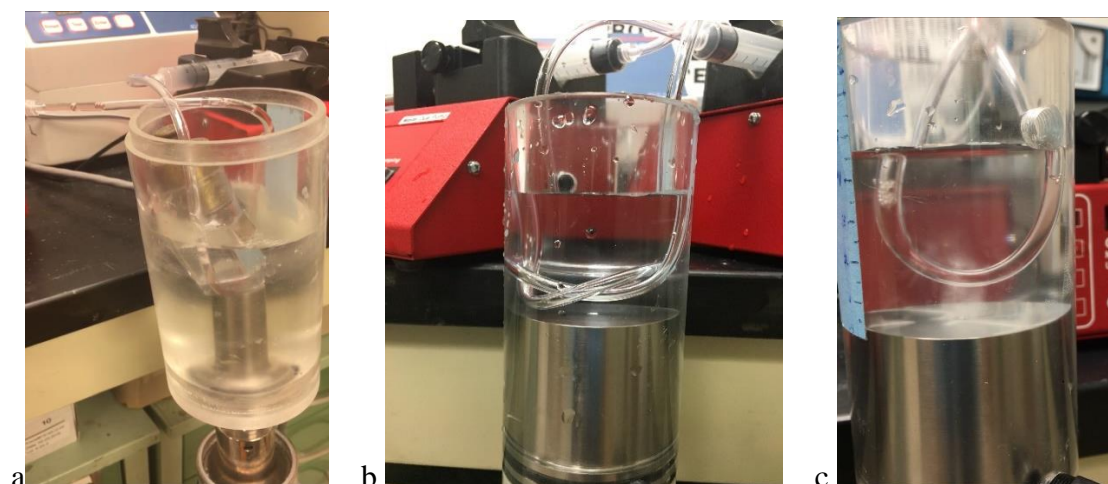


Figure 5: a) 19mm sonotrode horn (perpendicular setup) b) 60mm sonotrode horn (parallel setup) c) 60mm sonotrode horn (perpendicular setup)

Leukemia cell lines U937 and K562 were cultured and allowed to grow till they reach a total concentration between  $3 \times 10^5$  to  $1 \times 10^6$  cells/ml. For controls, the cells were stained with trypan blue and the concentration, viability and average size was measured using the cell counter. For stationary and moving sonication, 0.5ml of cells were taken in a syringe in aseptic conditions using a sterile needle. A tygon tube is taken and connected to the syringe and the cells are dispensed into it. The two ends of the tube are connected to the syringes respectively to form a closed network.

Two different configurations were analyzed. One in which the tube was placed above the sonotrode perpendicular to the field of sonication and the other in which the tube was coiled and placed parallel to the plane of sonication. The two syringes were connected to two pumps respectively where one is the master pump and the other is the secondary pump, which are synced together to form a to and fro motion. For stationary sonications, the pumps are not switched on and the cells remain static in the tube when exposed to sonication whereas in case of moving sonications, the pumps are switched on and the cells are in motion when exposed to sonication. The cells were exposed to 30 and 60secs of continuous sonication. The flow rate was

varied to see the difference in cell lysis that is the cells were analyzed at 25ml/min and 30ml/min flow rates. Once the cells are sonicated, they are stained with trypan blue and analyzed in the cell counter.

## 2.5 Analysis of cell morphology

The morphological changes in the cells when sonicated were examined in a light microscope. 10ul of cells were taken and placed on a microscopic slide and a glass slip was placed on it. This was viewed under 100x magnification. Cells were viewed before and after sonication and the differences were analyzed.

## 2.6 Annexin V/PI staining

The test was done to differentiate between the live cells and the cells undergoing apoptosis. Control, stationary and moving sonicated samples were taken to analyze using the apoptosis assay which uses Annexin V-FITC apoptosis detection kit for analysis. Two different stains were used Annexin V-FITC and PI, where Annexin V-FITC binds to the membrane PS and PI binds to the cellular DNA [g].

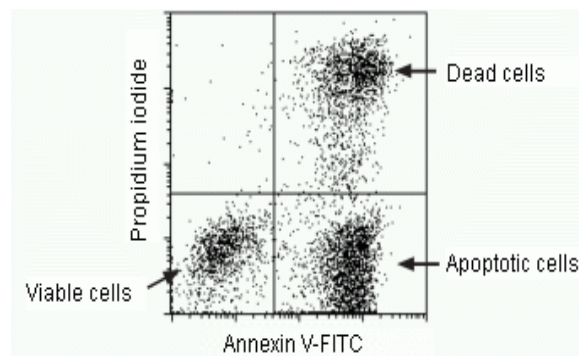


Figure 6: Sample graph of apoptosis assay  
Propidium iodide vs Annexin V-FITC [k]

In brief, the cells were exposed to sonications, that is stationary and moving and samples were prepared for the assay. Three samples were taken into consideration, control stationary and

moving. One positive control was made which contained most number of dead cells. This control was included to get the proper quadrant for the dead cells when viewed under flow cytometry. In the positive control, 70% ethanol was added to 0.5ml of cells which resulted in the death of all cells. Once the samples were ready, they were washed with PBS by gentle shaking or pipetting [g]. These cells were then resuspended in 200ul of Binding Buffer (1X). 5ul of Annexin V-FITC was added to 195ul of cell suspensions, mixed and incubated at room temperature for 10mins. The cells are again washed in 200ul of Binding Buffer (1X) and resuspended in 190ul of Binding Buffer (1X). 10ul of Propidium Iodide is added at the end and analyzed by FACS (Fluorescence-activated cell sorting). Flow cytometry was performed by BD Accuri C6 flow cytometer and the results were analyzed by BD Accuri C6 software [g].

## **2.7 Statistical Analysis**

The statistical analysis was done using t-test (Two samples assuming unequal variances). It is a data analysis tool in MS-Excel.  $P < 0.05$  was considered to be significant. Data was presented in the form of histograms of Mean and Standard deviation from at least 2 independent experiments.

### 3) RESULTS

The cell lines K562 and U937 showed an established growth cycle when cultured. Each cell line had a viability above 90% when used for experimentation. The average size of K562 cell lines was found to be between 25-27 micro meter and the average size of U937 cell line was in the range between 19 – 23 micro meters. The total concentration of the cells in the media was in the range  $3 \times 10^5$  to  $1.0 \times 10^6$  cells per ml for both the cell lines. The cell counter showed the graphs of cell size and gave the values of cell concentration and viability.

The cells seemed to be discoid in shape when viewed under the light microscope at 100X magnification. The images show the size difference between u937 and k562 cell lines where the k562 are a lot bigger than the u937. Both these cell lines had their average size much greater than the normal whole blood cells thus making it easy to differentiate between the normal and leukemia cells.

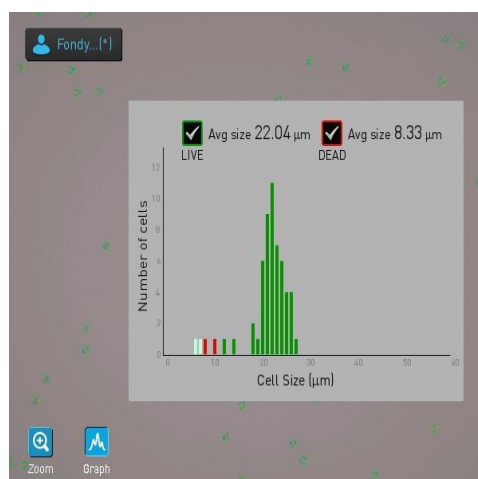


Figure 7: U937 Cell counter data

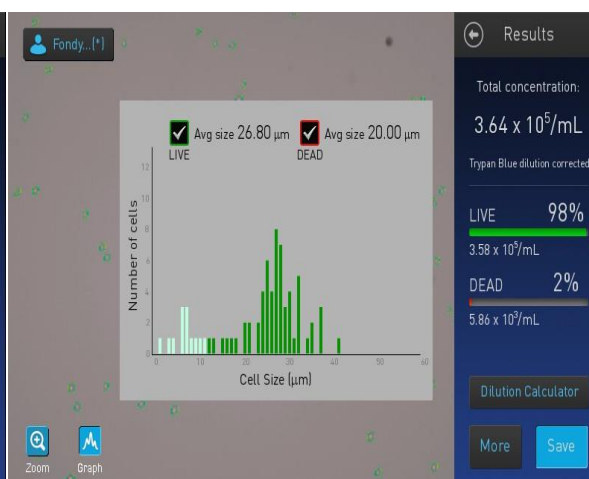


Figure 8: K562 Cell counter data

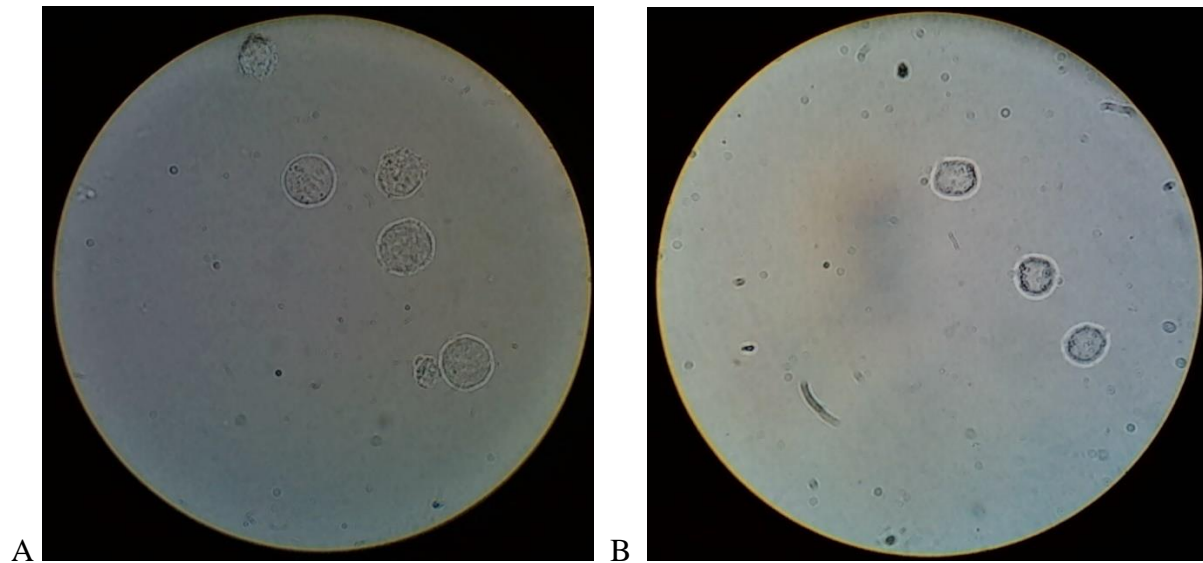
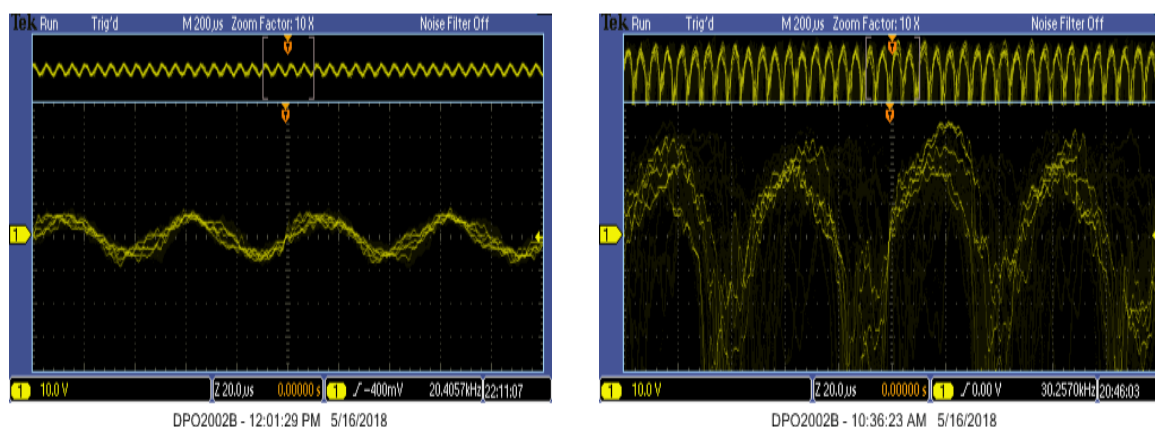


Figure 9: Light microscope images under 100X magnification A) K562 B) U937

### 3.1 Ultrasound frequency and cavitation readings

The parameters were fixed for applying sonications to the leukemia cell lines. A high intensity low frequency ultrasound was delivered to the cells to result in cavitation and selective cell destruction. 20khz frequency was used and two different diameter sonotrode, that is 19mm and 60mm sonotrode horns were used which showed two different intensities when measured by cavitation meter. 19mm sonotrode horn gave an intensity of 200 cavins which was equivalent to  $0.26 \text{ W/cm}^2$  whereas the 60mm sonotrode horn gave an intensity of 250 cavins which was equivalent to  $0.325 \text{ W/cm}^2$ . This showed that the 60mm sonotrode horn delivered a higher intensity than the 19mm one that is the cavitation was higher in the former horn.



A)

B)

Figure 10: Oscilloscope images for 20kHz system at 30% amplitude A) 19mm sonotrode horn  
B) 60mm sonotrode horn

### 3.2 Extracorporeal setup for sonications

The syringe pumps were configured and synchronized to one another for a to and fro motion. Moving control was not included in the experiments because moving alone didn't cause any damage to the cells. The control used was the cells which are not in motion and not sonicated. Stationary sonication was analyzed by keeping the cells in the tube without turning on the pumps, that is the cells were static. There were two configurations considered, one perpendicular to the field of sonication and another planar to the field of sonication. 0.5ml of cells were there in the tube during sonications. It was seen that when the cells are stationary and exposed to a continuous sonication for 60 secs, there was a decrease in the cell viability and concentration for both the configurations. The average decrease in live cell viability was in the range of 5-10% decrease. There was a slight decrease in the average size as well when they were sonicated. The experiments were repeated and there were similar results obtained. The viability ranged from 80 to 90% live cells and the average size was 24μm and 19-21μm for k562 and u937 respectively.



For moving sonication, there were changes in parameters and configurations as well. They were also accessed for perpendicular and planar configurations and both showed similar results. There were changes in the result when accessed for varying flow rates. Two flow rates were analyzed that is 25ml/min and 30ml/min. The time of exposure was kept the same that is 60secs of continuous sonication. It was seen that when the flow rate is 25ml/min, the cells pass three times through the sonication field whereas when the flow rate is 30ml/min, the cells pass 4 times through the field of sonication in 60secs but in case of 25ml/min, as the cells are moving slowly they are exposed to the sonication field for a longer time in one cycle. When the viability was accessed, it showed a greater decrease when the flow rate was 30ml/min in comparison to 25ml/min. Overall the cell viability went down about 25 to 30% and in some cases even below. The average size and concentration also showed a greater decrease in comparison to the control and stationary sonication results. The viability of the live cells was in the range of 70-75% and in one two experiments it went down till 30% in the case of k562 cell line. The U937 cell lines had live cell viability in the range of 55-70%. The average size of k562 cells went down in the range of 21 to 24um and the average size of u937 was in the range of 16 to 19um. There was a big difference in the total concentration in many experiments for both the cell lines. The total concentration decreased by a huge number in moving sonication as compared to control and stationary.

The sonications using 60mm sonotrode horn showed more damage in both stationary and moving experiments as compared to the 19mm diameter sonotrode horns. Only perpendicular configuration was analyzed in case of 19mm sonotrode as optimization of planar configuration was difficult.

### 3.3 Analysis of K562 when moving and not sonicated

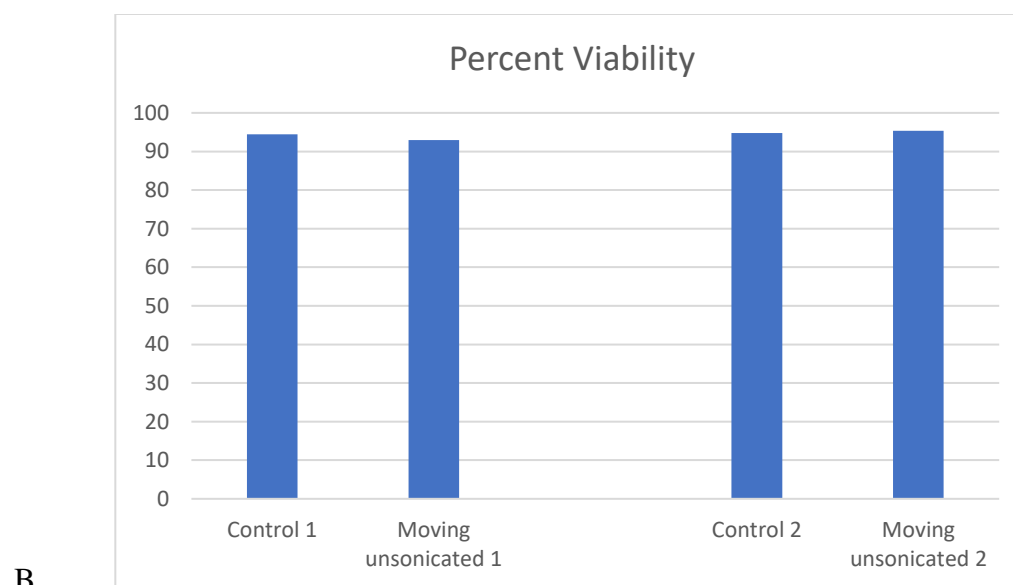
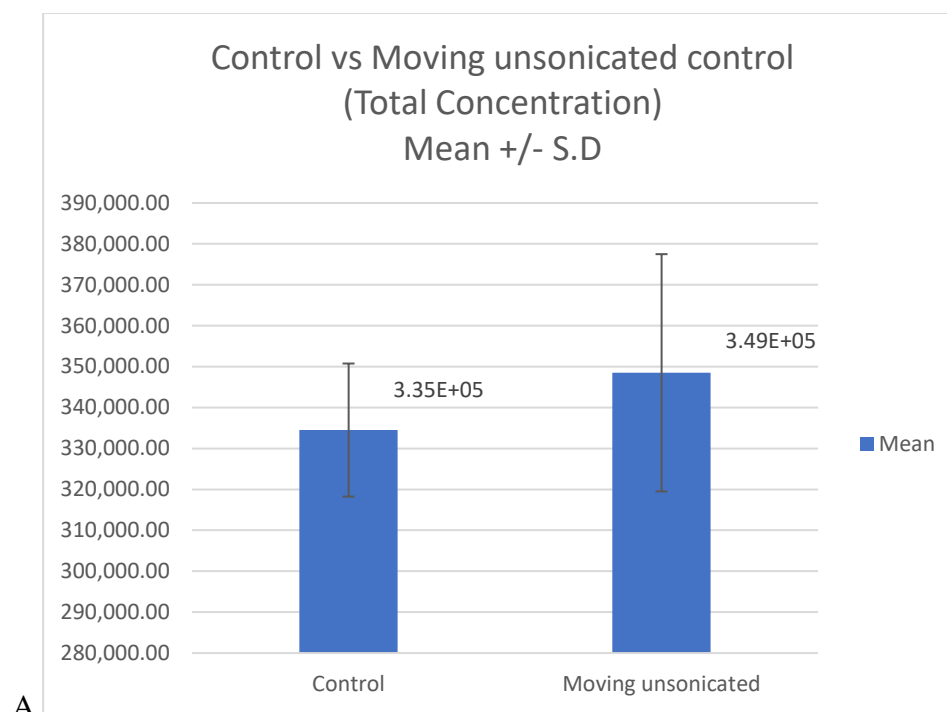


Figure 11: Effect of movement alone on K562 (n=2) A) Difference in total concentrations B) Mean +/- S.D and C) Percent viability.  $P > 0.05$

### 3.4 Analysis of U937 when moving and not sonicated

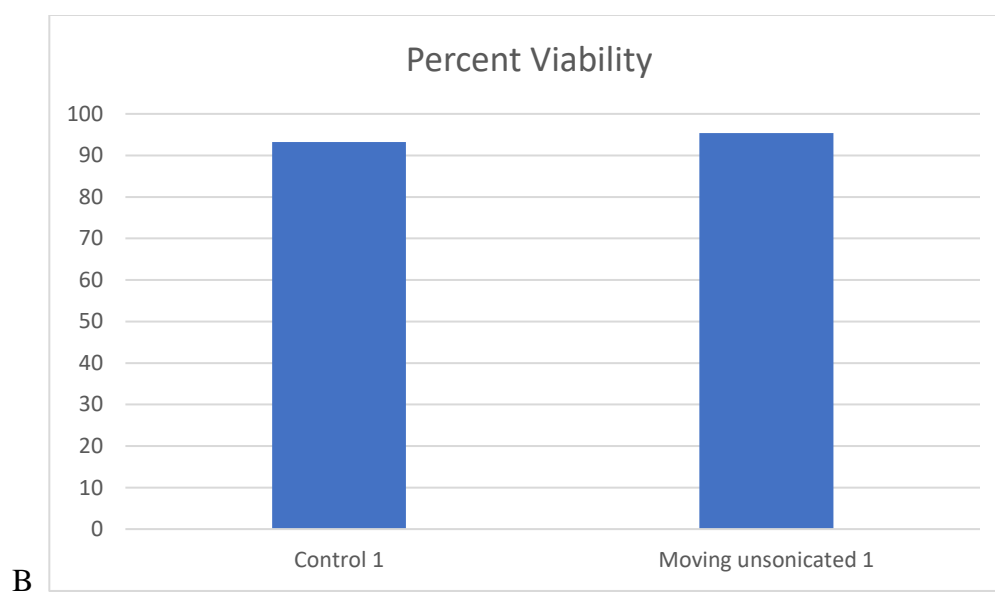
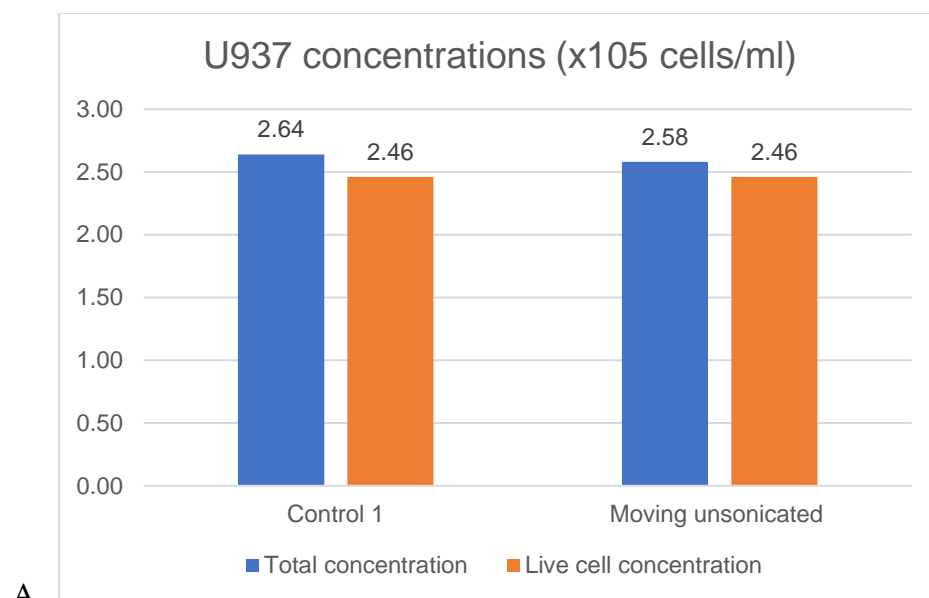


Figure 12: Effect of movement alone on U937 A) Difference in total concentrations  
B) Percent viability

3.5 Analysis of sonications of K562 using 19mm sonotrode horn (20khz at 30% amplitude  
0.26W/cm<sup>2</sup> intensity)

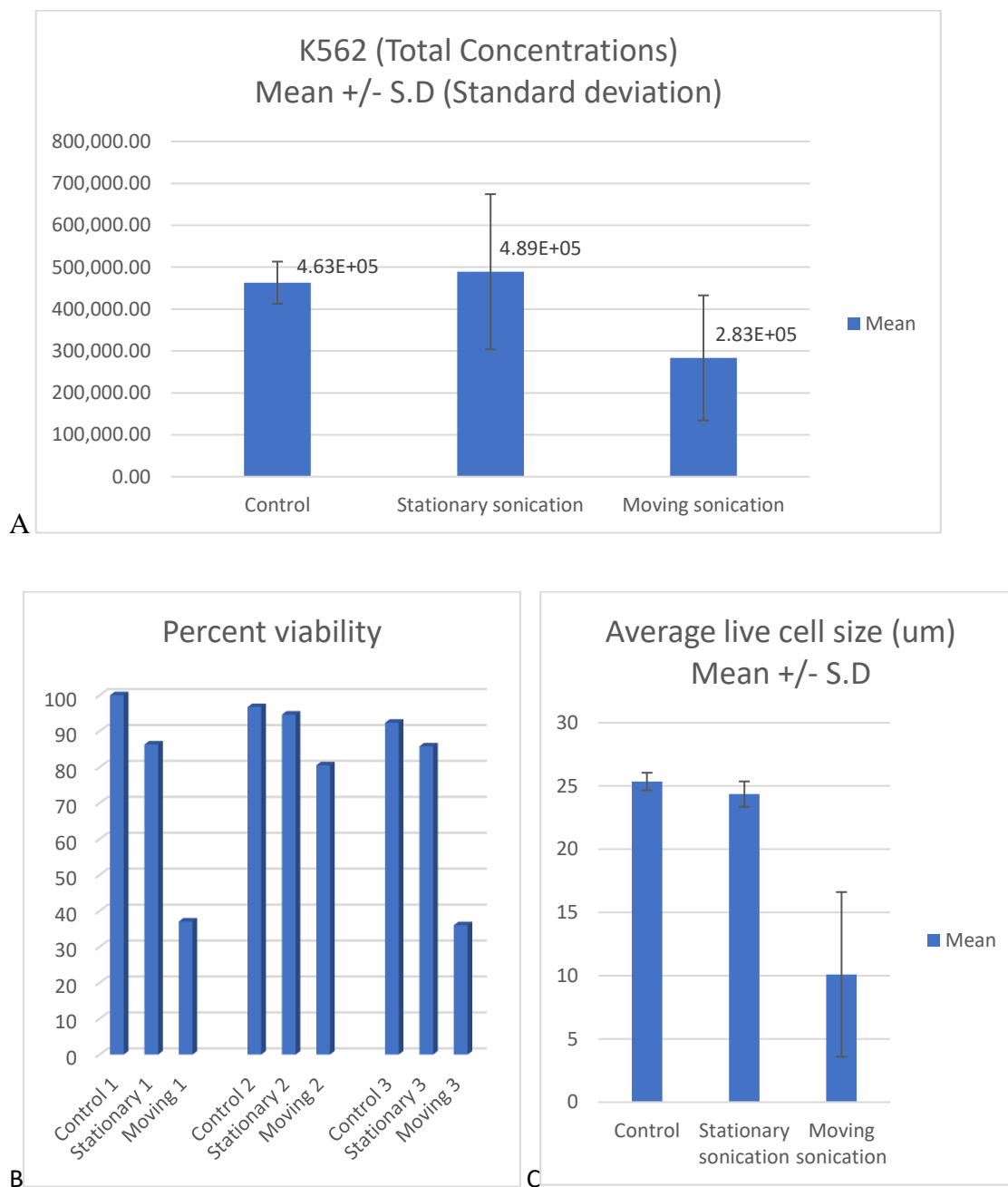


Figure 13: Effects of continuous low-frequency ultrasound on K562 using 19mm sonotrode horn A) Total cell concentration B) Percent viability and C) Changes in the average live cell size (n=3). P>0.05 for control vs stationary sonication and p<0.05 for control vs moving sonication

3.6 Analysis of sonications of U937 using 19mm sonotrode horn (20khz at 30% amplitude  
0.26W/cm<sup>2</sup> intensity)

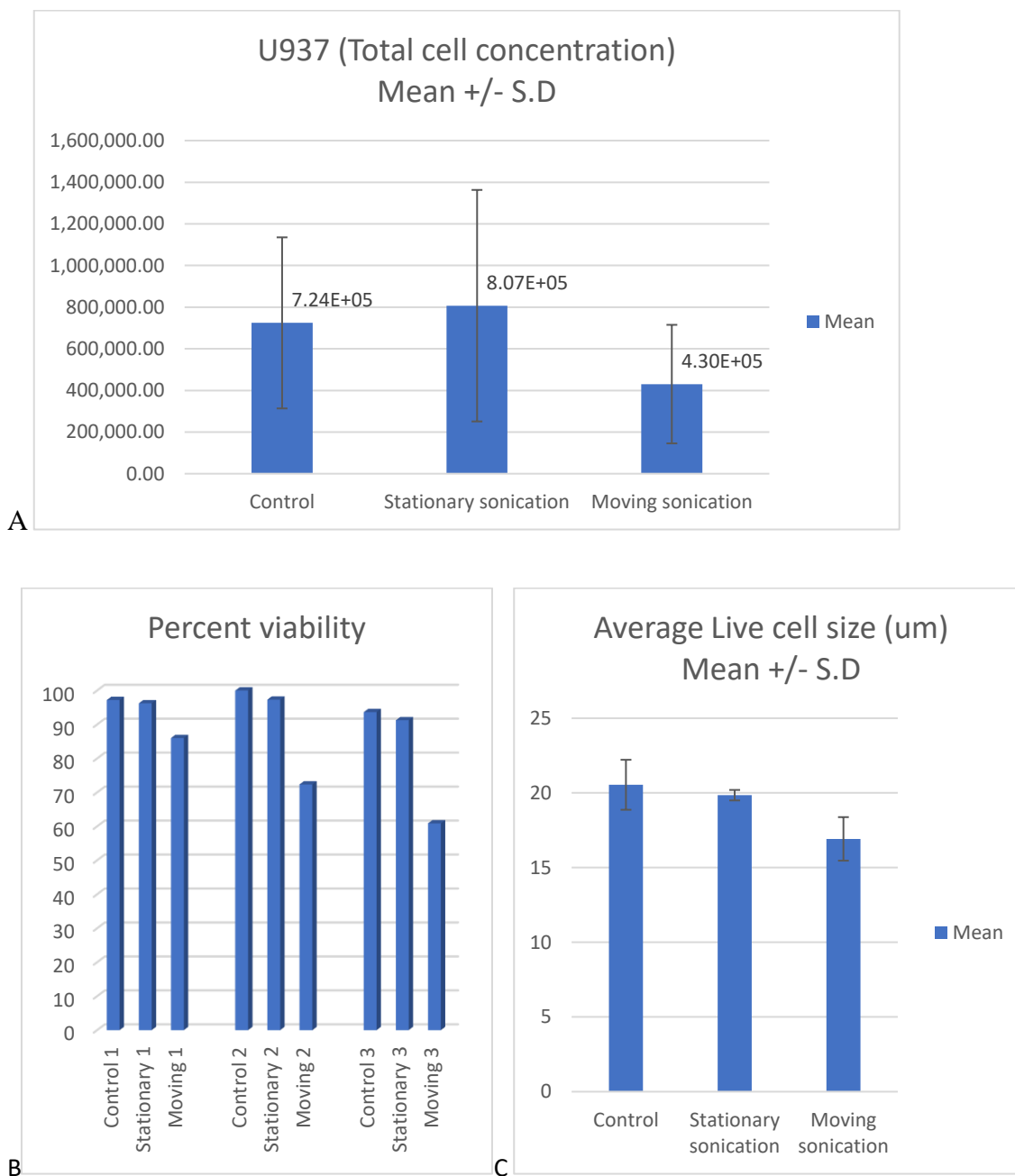


Figure 14: Effect of low frequency ultrasound on U937 using 19mm sonotrode horn A) Total cell concentration B) Percent viability and C) Changes in the average live cell size (n=3). P>0.05 for control vs stationary and for control vs moving

3.7 Analysis of Sonications of K562 using 60mm sonotrode horn (20khz at 30% amplitude  
0.325W/cm<sup>2</sup> intensity)

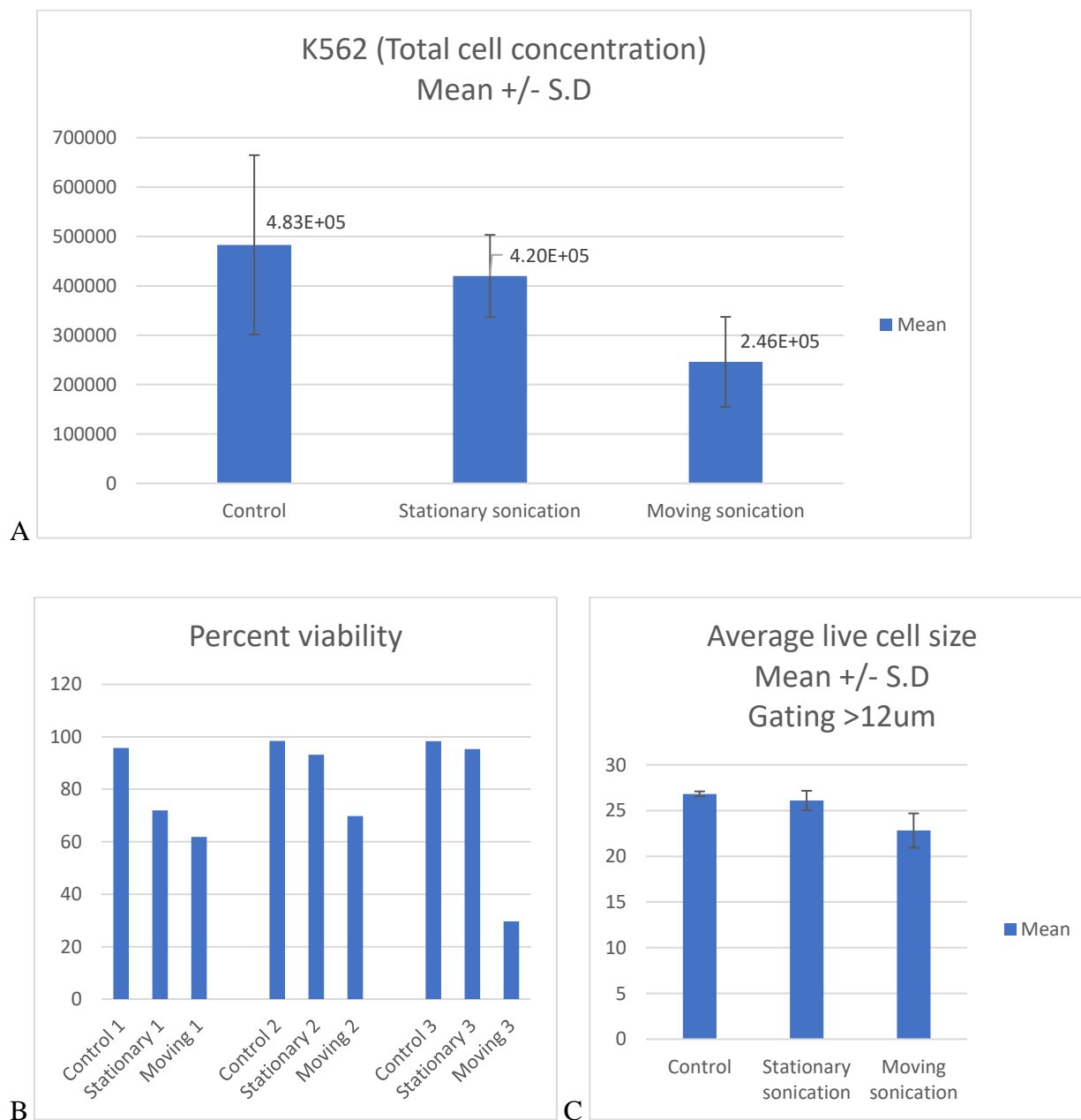


Figure 15: Effects of low-frequency ultrasound on K562 using 60mm sonotrode horn A) Total cell concentrations of samples B) Percent viability and C) Changes in the average live cell size (n=3)  
p>0.05 for control vs stationary and p<0.05 for control vs moving

3.8 Analysis of sonications of U937 using 60mm sonotrode horn (20khz at 30% amplitude  
0.325W/cm<sup>2</sup> intensity)

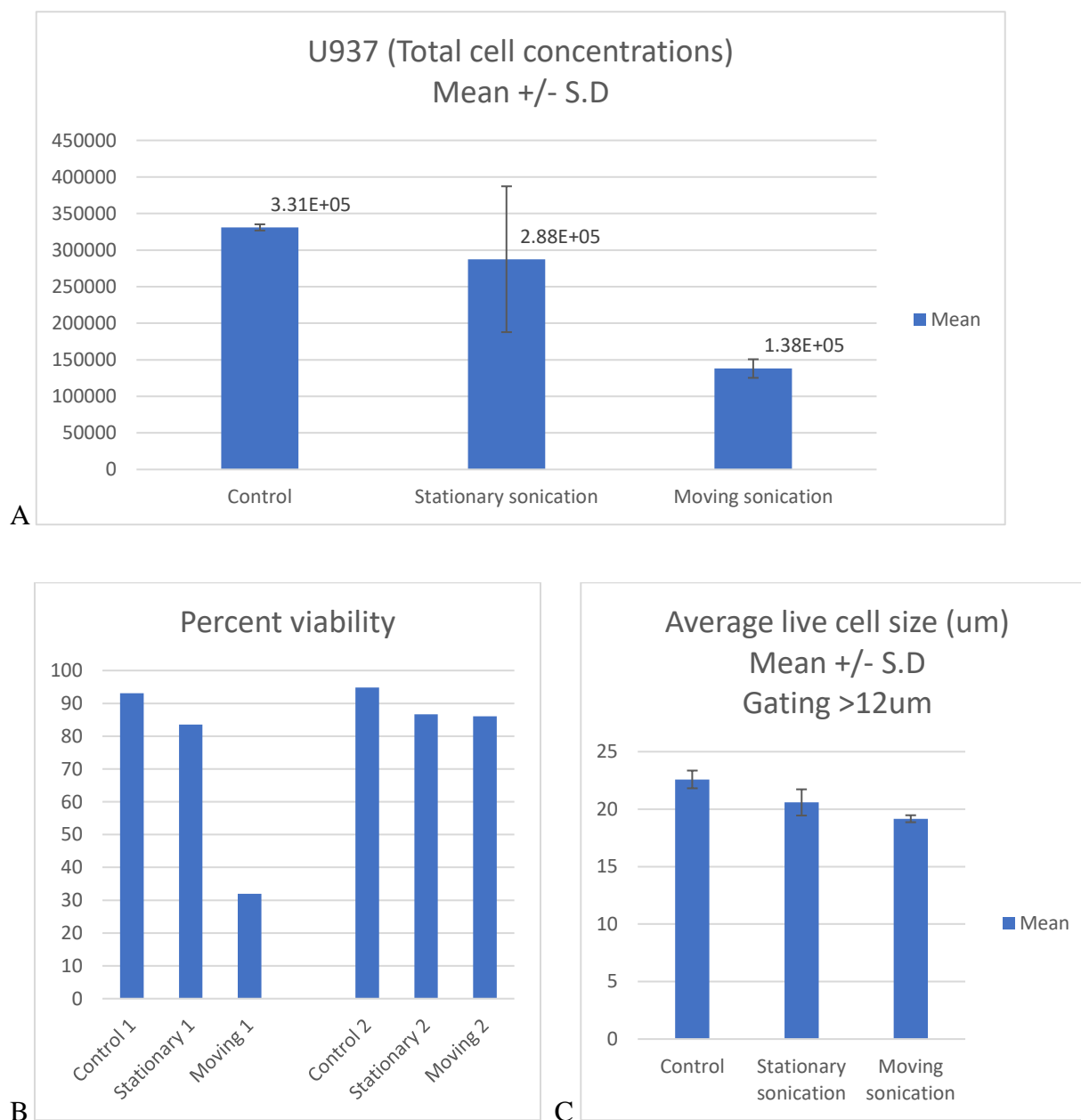


Figure 16: Effects of low-frequency ultrasound on U937 using 60mm sonotrode horn A) Total cell concentration of samples B) Percent viability and C) Changes in the average live cell size (n=2)  
p>0.05 for control vs stationary and control vs moving

3.9 Analysis of sonications of K562 (Change in the flow rate) (60mm horn) (20khz at 30% amplitude, 0.26W/cm<sup>2</sup> intensity)

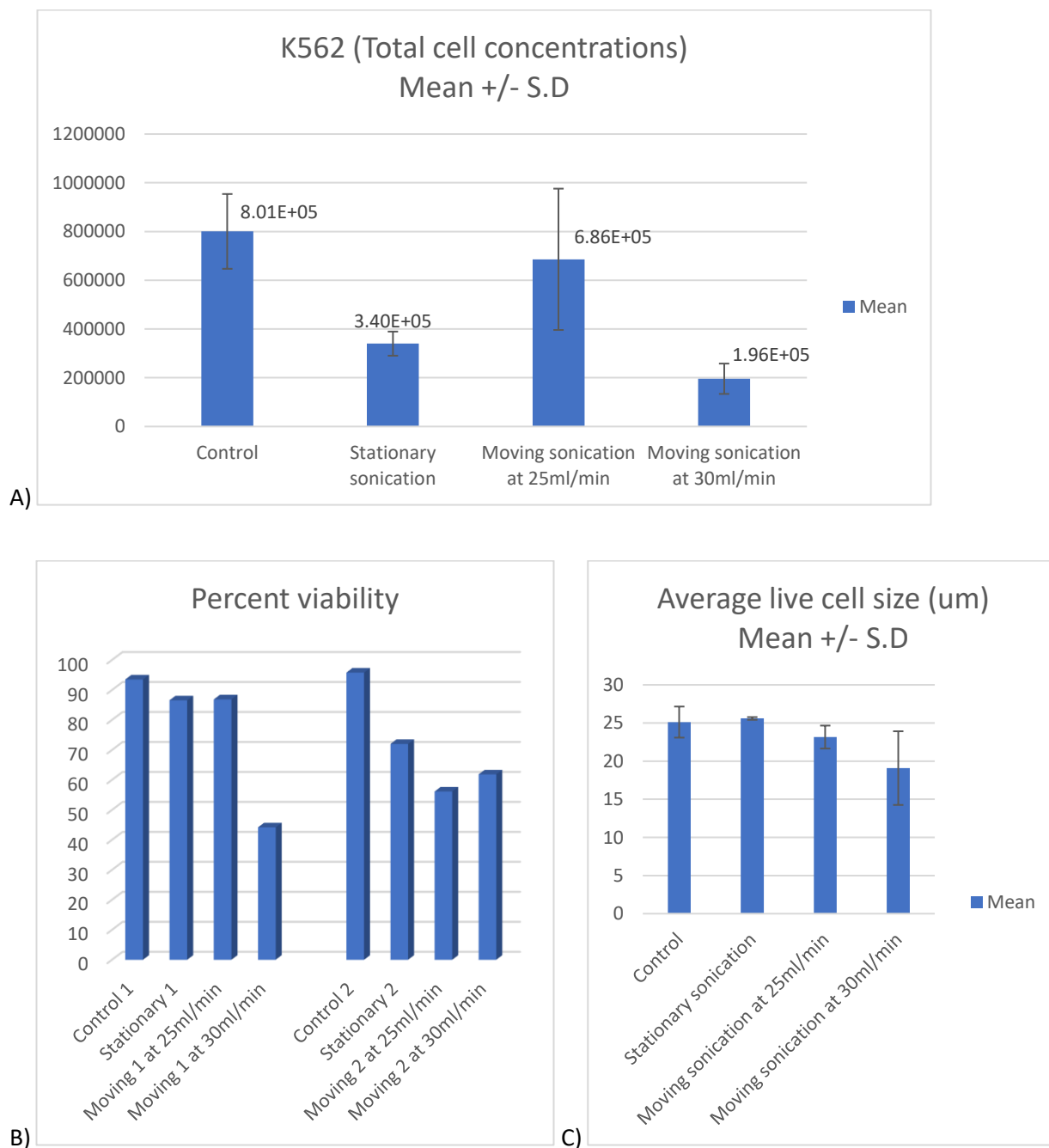


Figure 17: Effects of change in flow rate on sonication of K562 A) Total cell concentrations of samples B) Percent viability and C) Average live cell size (n=2).  $P > 0.05$  for control vs stationary, control vs moving at 25ml/min and control vs moving at 30ml/min



3.10 Analysis of sonications of U937 (Change in the flow rate) (60mm horn) (20khz at 30% amplitude, 0.26W/cm<sup>2</sup> intensity)

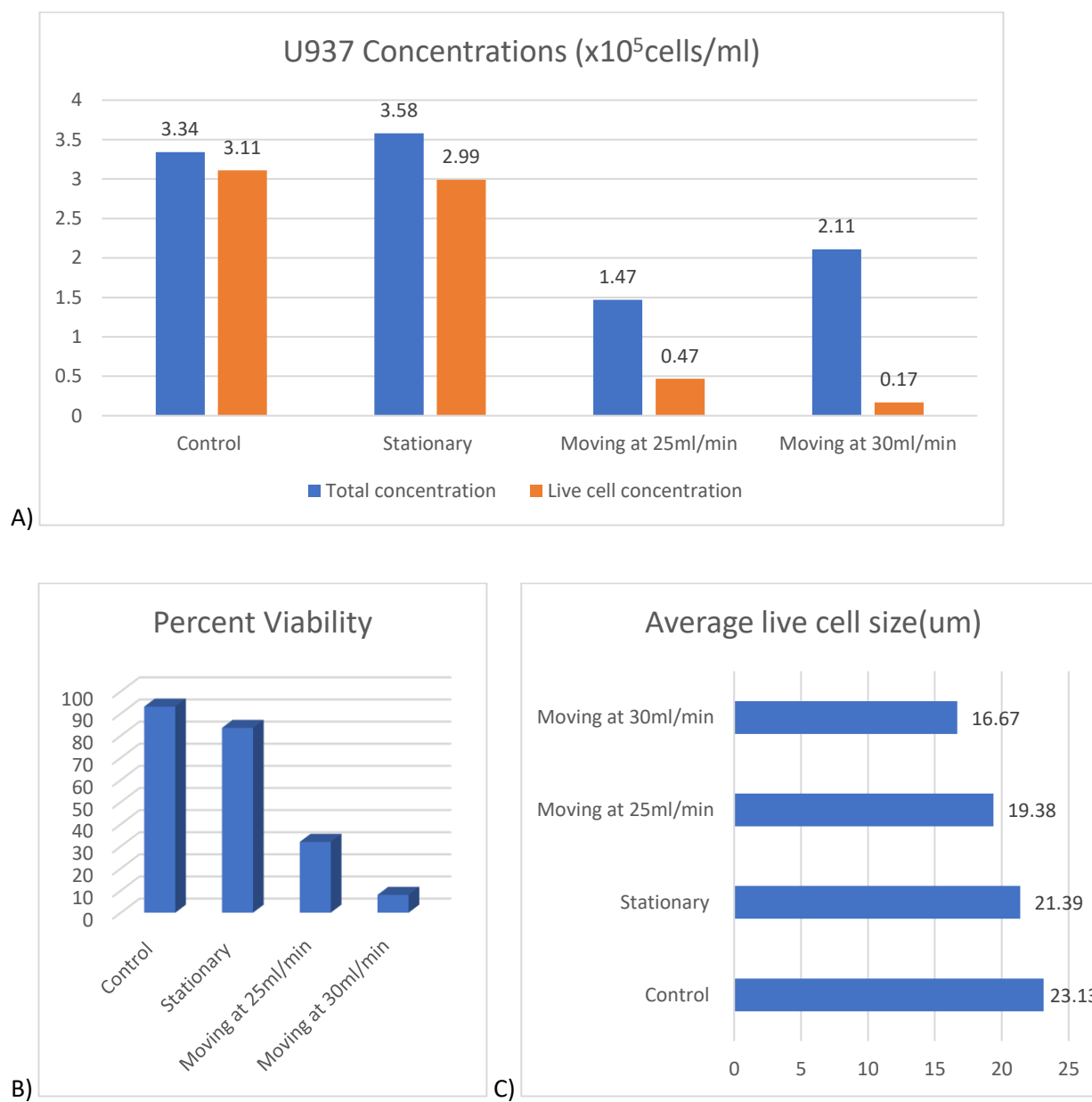
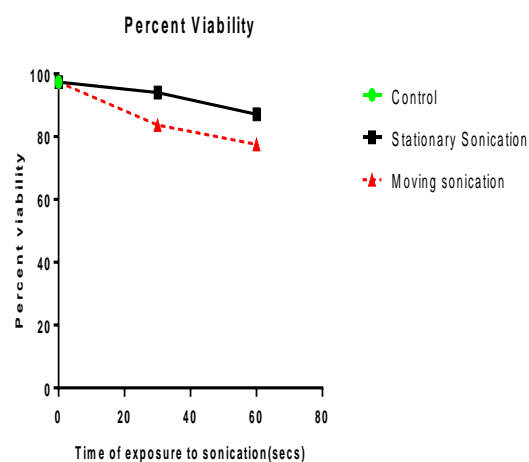
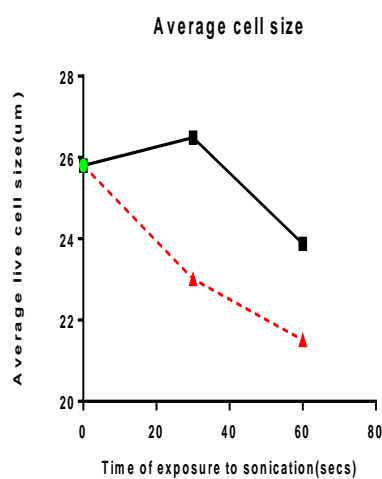
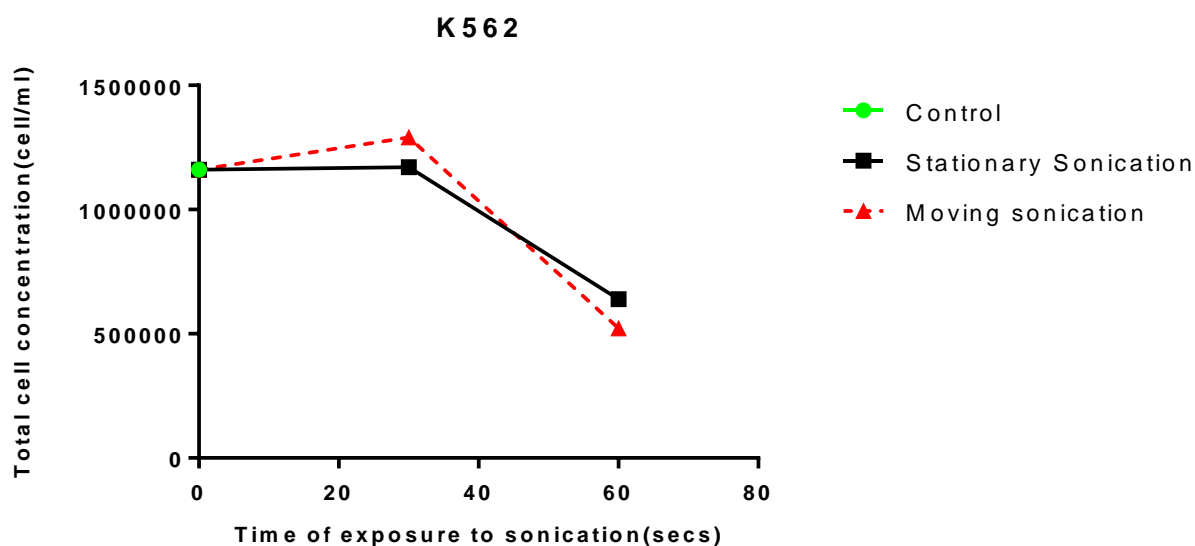


Figure 18: Effects of change in flow rate on sonication of U937 A) Total cell concentrations of samples B) Percent viability and C) Average live cell size

3.11 Analysis of sonications of K562 (Varying time of exposure) (60mm horn) (20khz at 30% amplitude, 0.26W/cm<sup>2</sup> intensity)

A)



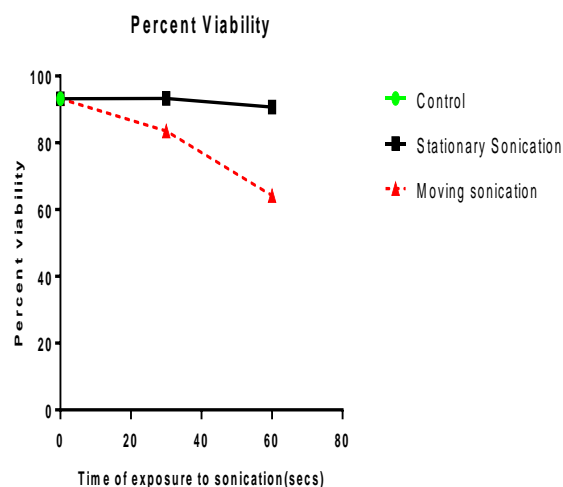
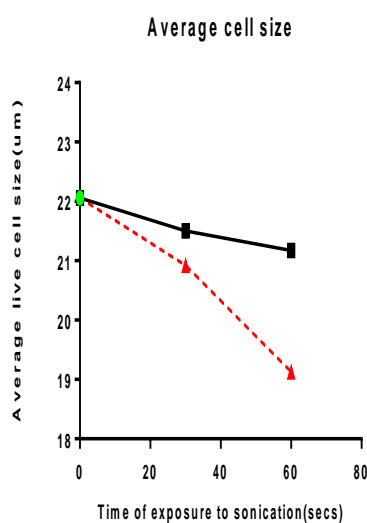
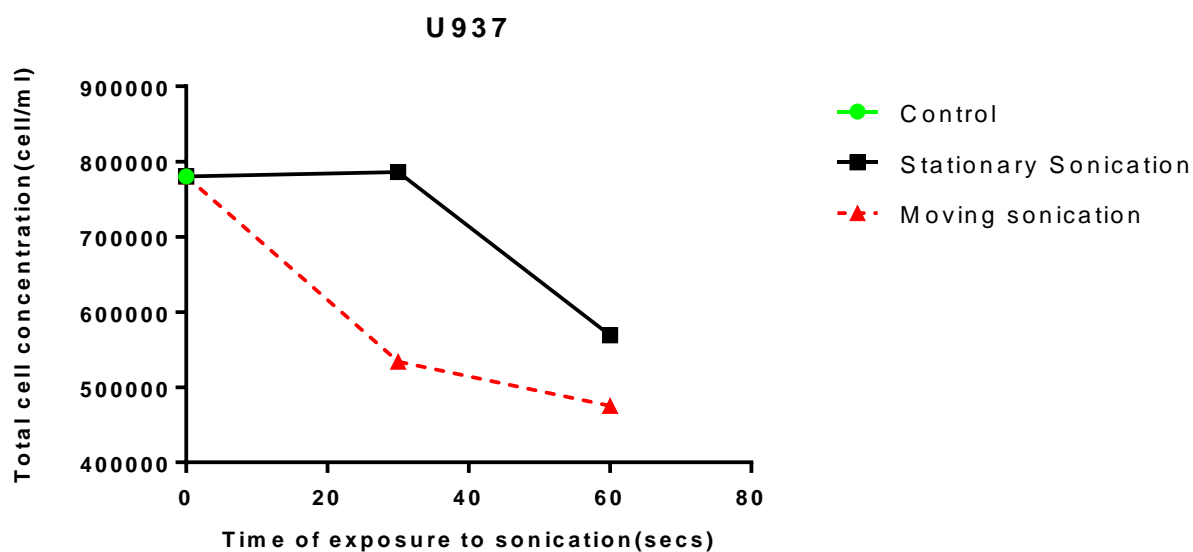
B)

C)

Figure 19: Analysis of varying time of exposure of sonication on K562 A) Total cell concentration of samples B) Average live cell size and C) Percent viability

3.12 Analysis of sonications of U937 (Varying time of exposure) (60mm horn) (20khz at 30% amplitude, 0.26W/cm<sup>2</sup> intensity)

A)



B)

C)

Figure 20: Analysis of varying time of exposure of sonication on U937 A) Total cell concentration of samples B) Average live cell size and C) Percent viability

### 3.13 Morphology of lysed cells

When the cells were viewed under the microscope, there was an evident difference in cell size and there was a decrease in the cell concentration as seen under the microscope. Some cells were seen to undergo apoptosis as their regular circular structure seemed to be distorted. Overall the cells seemed to show damage in terms of cell size and cell concentration. The decrease in the live cell concentration was also visible in the images from the cell counter when checked for the viability.

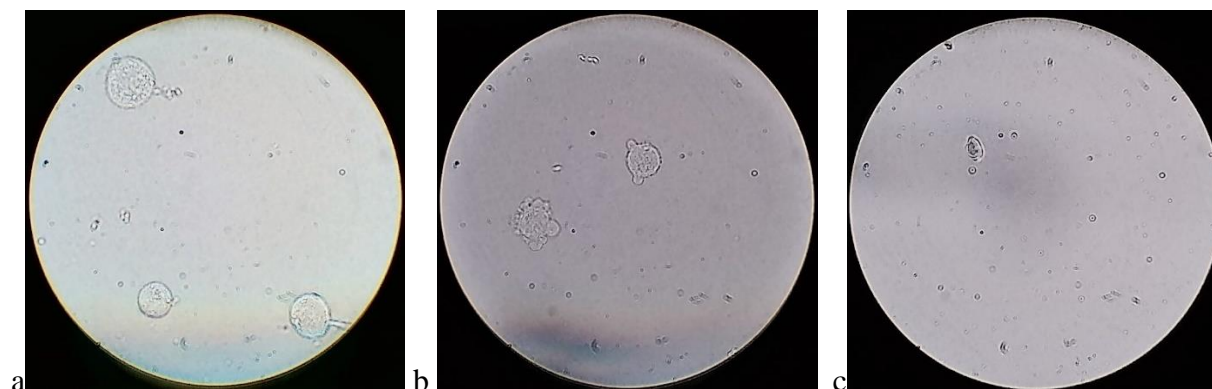


Figure 21: Microscopic images of K562 a) Control b) Stationary sonication and c) Moving sonication

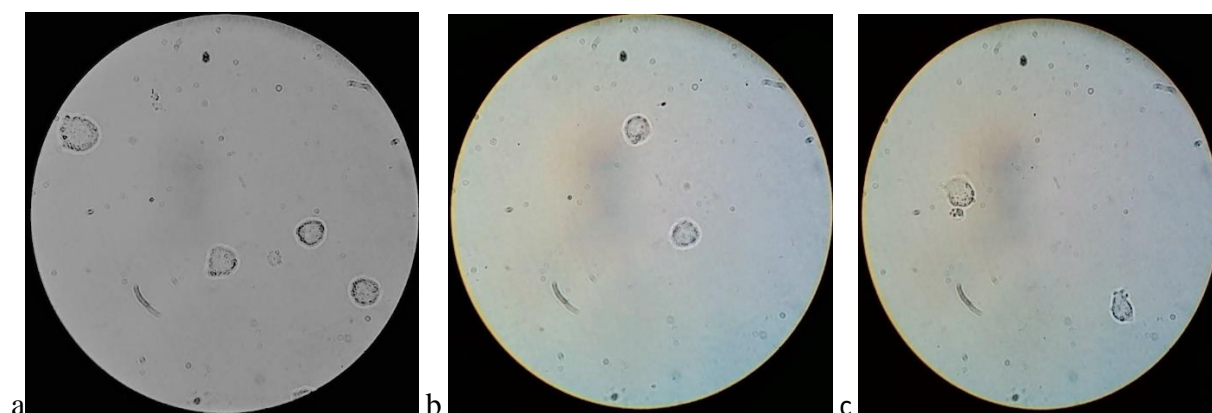


Figure 22: Microscopic images of U937 a) Control b) Stationary sonication and c) Moving sonication

### 3.14 Annexin V/PI staining

Annexin V-FITC apoptosis assay was performed and it gave a graph differentiating the live cell, dead cells and cells undergoing apoptosis. When the cells undergo apoptosis, it starts losing its membrane structure that is the PS(phosphatidylserine) is translocated to the outside thus exposing it to the outer environment, thus helps in differentiating the apoptotic cells to the normal cells. The Annexin V having a high affinity for PS attaches to the exposed PS in the cells undergoing apoptosis <sup>[f]</sup>. The PI being a flow cytometric viability probe is used to differentiate viable and non-viable cells. The viable cells do not allow PI to get into them whereas non-viable cells with damaged membranes are permeable to PI. Thus, when the cells stain positive for both Annexin V and PI, they are on the last stage of apoptosis or are dead and when they stain negative for both they are live cells. When they stain positive for Annexin and negative for PI they are undergoing apoptosis <sup>[f]</sup>. When the samples were analyzed in flow cytometry, the control showed maximum live cells that is negative FITC Annexin V and PI staining whereas in case of stationary and moving sonication samples there were more dead cells, moving samples having the most in comparison to control and stationary samples. There were very few cells in the quadrant which had a positive Annexin V staining and negative PI staining, that is the cells after sonication were dead rather than undergoing apoptosis.

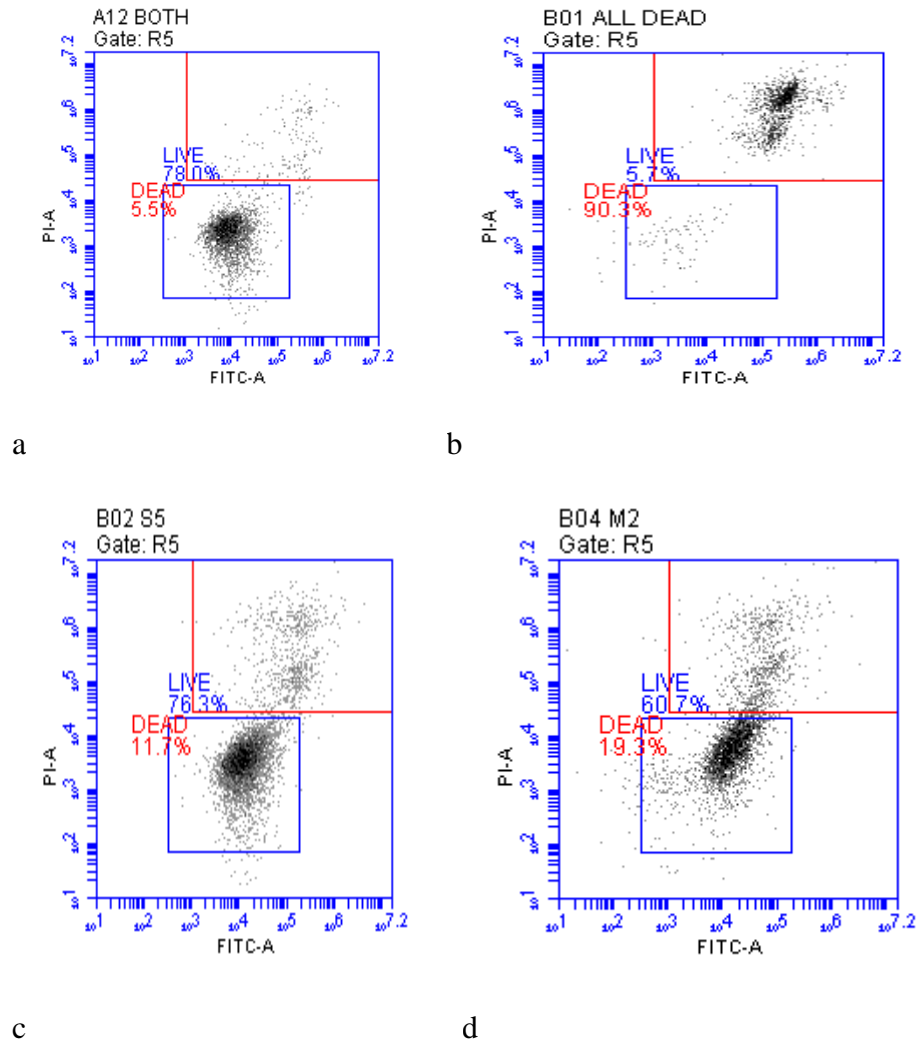


Figure 23: Results obtained from flow cytometry after apoptosis assay of K562 a) Control b) Positive control c) Stationary sonicated d) Moving sonicated

#### 4) **DISCUSSION**

Research on cancer biology and cancer medicine is designed to improve treatment outcomes and to provide long-term cancer survival with acceptable side reactions to the treatments. Cancer pharmacological treatments are most commonly designed to work at the cellular level based on cancer and host biochemistry or immunobiology. Our current research is designed to use physical treatment protocols rather than drug therapy or immunotherapy. Physical approaches such as X-ray therapy, hyperthermia, thermal ablation, and phototherapy along with surgery itself are also used in cancer management. We employ pulsed and continuous low-frequency ultra-sound between 15 kHz and 67 kHz as a physical approach to preferential damage of untreated and treated cancer cells based on size and cell biological differences between normal and neoplastic cells.

One important variable in attempting to use low-frequency ultra-sound for the preferential destruction of cancer cells is the effect of cell movement through the sonic field. Is response to ultra-sonic treatment affected by cell movement, including the direction, volume of flow, velocity of flow, and configuration of sonic target vessel. We have determined that low frequency ultra-sound at 20khz can damage leukemia cells with acceptable side effects to normal leucocytes and red blood cells when the intensity was kept between 0.25 – 0.35 watts/cm<sup>2</sup>. The flow rate for the experiments were kept at 30ml/min because it was quite like the speed at which the blood flows in the arteries. The diameter of the tygon tubing used was 0.125inches (3.175mm) which was close to the diameter of the arteries. When using 19mm sonotrode horn, it was seen that the cells closer to the sonotrode horn receive maximum sonication in comparison to the cells at the vicinity, which suggests that not all cells are receiving equal amounts of sonication when accessing stationary sonications. In case of moving sonication, all the cells receive equal sonication, as they will pass through the field at least once when they are in motion and the sonic effects are averaged for the

total cells in the population. Using a 19mm sonotrode horn didn't give comparable results between stationary and moving sonications because the target exposure configuration did not allow for full sonication of all the cells. Thus, 60mm sonotrode horns were used for these experiments because all the cells were affected by sonications in the larger field. The setup when kept in perpendicular configuration was shown to have a less damage in comparison to the parallel configuration, the reason being the cells at the vicinity were not being affected. When kept in a parallel configuration, all the cells are at the same distance from the sonic source and are exposed to the field of sonication equally both in case of stationary and moving. In case of changing the flow rate, the 30ml/min flow rate seemed to show maximum damage in comparison to the one at 25ml/min. The main reason is that even though when the speed is low, the cells pass through the field thrice in comparison to when the cells are moving at 30ml/min, where they pass through the field 4 times in 60secs. So, by getting the same results for the experiments, it is seen that the number of cycles or the number of times the cells pass through the sonication does **affect** their damage. When the cells are stationary, the cavitation does cause damage but comparatively less than that caused when they are moving. This may help us to design approaches for the in vivo application of low frequency ultra-sound. For in vivo treatments the cells will be moving inside the blood in the body and when sonications are applied the cells will be in the state of motion.

It is also seen that when the cells are exposed to sonication, their average cell size changes. This proves that when sonications are applied, it can knock off the bigger diameter cells and thus decrease the average size of live cells. The leukemia cells being bigger in size as compared to normal whole blood cells are an easy target when they are in motion as hypothetically they will be more affected by the cavitation in comparison to smaller cells. When apoptosis assay was performed on the samples, it showed two separate colonies of live and dead cells respectively.



Based on the graphs obtained it was seen that the dead cell colony was much bigger in the moving sonications in comparison to the control and the stationary. The results showed that the low frequency ultrasound caused necrosis of the leukemia cells and helped to decrease their viability.

Overall the experiments seemed to put up a point that the leukemia cells are more sonic sensitive when they are in motion in comparison to when they are stationary, and that the viability of the cells went down considerably in case of moving sonications. Further experiments will be lined on mixing the human whole blood with the leukemia cells and then sonicating them, to see how the whole blood cells react to the sonications and if they are not damaged more in comparison to the leukemia cells that will be a step forward of using this study for in vivo work. Hypothetically, the whole blood cells being always in motion will be less affected by the sonication in comparison to leukemia cells which will be affected more as they are larger in diameter and not free flowing. Studies on stationary mixed cells has shown that the whole blood cells are less damaged but there is also not much damage of the leukemia cells.

For future studies, a more concise and compact system is to be created to produce similar results as seen in the extracorporeal setup. These results can be used for formulating the time of exposure of sonication which will be required when working on mice models. Also, research can be done on using sequential sonications at intervals to try causing more damage to the leukemia cells. This is because when the leukemia cells are sonicated, there is a considerable damage but after 24hrs they tend to regrow and cover up for the lost/damaged cells. When the cells undergo sequential sonication at say 6hr or a 12hr interval, the cells don't get the chance to regrow back and there is a chance of maximum damage which can be achieved. Also, an apheresis machine can be utilized in case there is more damage caused by the sonication on the skin or adjacent tissues. Apheresis is a technique used for the extraction of various components of the blood by

the withdrawal of the blood, followed by density gradient centrifugation which helps to separate out the components <sup>[m]</sup>.

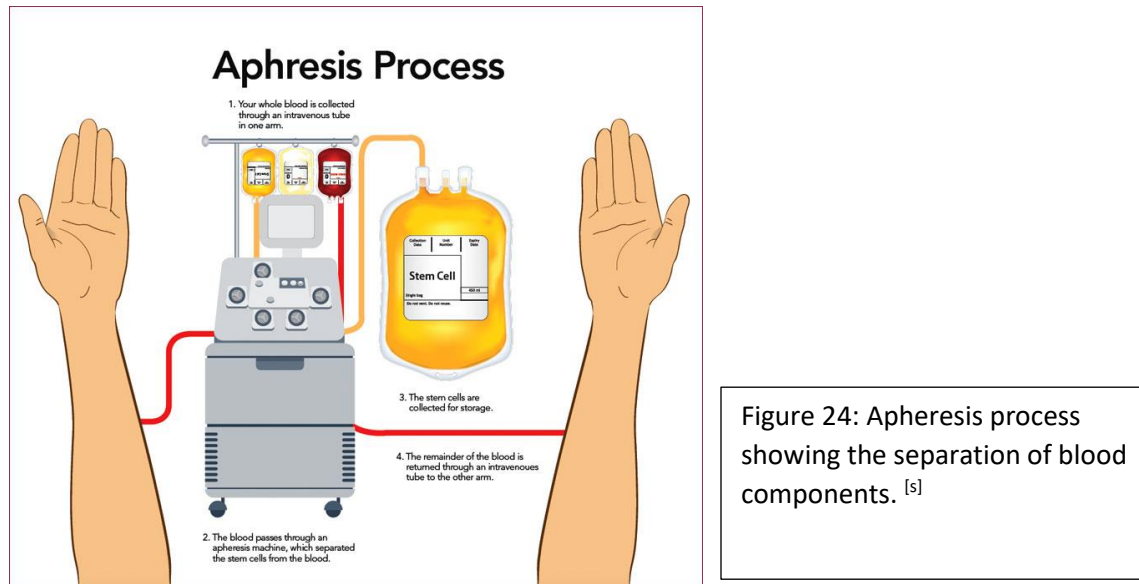


Figure 24: Apheresis process showing the separation of blood components. <sup>[s]</sup>

Hypothetically, this system can be used to provide a setup to perform sonications in vitro. The blood is drawn out of a patient suffering from leukemia and before it undergoes centrifugation, the setup is such that the cells are sonicated first up to a point where the blood cells are least damaged. The blood then undergoes centrifugation and the debris and dead cells are removed out and the rest of the blood components are put back into the body. This can be done at an interval till the time the leukemia cell levels have decreased a considerable amount. This method won't be able to eradicate the disease or completely cure the patient, but it will give a considerable amount of time for the patient. There have been studies where the concept of apheresis for reducing the number of leukocytes in the peripheral blood. It is called as leukapheresis and this concept can be incorporated into this where leukapheresis and sonications can be combined to cause an effective treatment option for leukemic patients. <sup>[t]</sup>

The use of various chemotherapeutic agents should be examined in combination with low frequency ultrasound to induce cell lysis. These two treatment modalities may have a synergistic effect on the regulation and reduction of leukemia in vivo. <sup>[u]</sup>

## **5) CONCLUSION**

Recent advancement in research has made it possible to come up with various treatments for cancer which will not only help to reduce their spread but also move on to eradicate them.

Ultrasound treatment can be a great step forward towards formulating a non-invasive method to fighting leukemia and can help to move on to a cure in combination with various chemotherapeutic agents. If ultrasound succeeds in reducing the amount of cancer before a surgery or before any other treatment, it will be convenient for the doctors as well to treat the patient and it will help to prevent its spread.

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## 7) CV/ RESUME

<b><u>EDUCATION:</u></b>	<p><b>Syracuse University</b>, Syracuse, NY Aug 2016 – Present  Masters of Science, Bioengineering <b>GPA: 3.58</b>  <b>Relevant courses:</b> Biomechanics, Physical Cell Biology, Biology of Cancer, Principles of Tissue Engineering, Biomaterials and Medical Devices, Biomanufacturing, Stem Cell Engineering</p> <p><b>Thadomal Shahani Engineering College</b>, Mumbai, India Aug 2012 – May 2016  Bachelor of Engineering, Biotechnology <b>GPA: 3.40</b>  <b>Relevant courses:</b> Cell Biology, Immunology, Stem Cell Biology, Genetic Engineering, Biochemistry, Biophysics, Plant and Animal Tissue culture, Bioinformatics, Enzyme Engineering</p>
<b><u>SKILLS:</u></b>	<p><b>Software:</b> MS-Office Suite (Excel, Word, PowerPoint), RASMOL, Pubmed  <b>Languages:</b> MATLAB, R  <b>Lab skills:</b> Pipetting, Centrifugation, Cell culturing, Basic training on mouse handling, Media preparation, suspension culture, Working in Hood (aseptic condition), DNA extraction, CFU counting, chromatographic techniques, working on cellometer, Spectrophotometer, passaging of cell lines, staining of cells, Use of microscopes for viewing cells, Actin staining</p>
<b><u>EXPERIENCE:</u></b>	<p><b>Syracuse University</b>, Syracuse, NY Jul 2017 – Present  Graduate Research Assistant, Department of Biology (Dr. Fondy's Lab)  <ul style="list-style-type: none"> <li>• Researched and modeled a system to access the effects of low frequency ultrasound on flowing cell lines and compared the results to stationary cell lines</li> <li>• Varied the flow rate of cells and frequency of ultrasound and accessed its effects</li> <li>• Performed apoptosis assays, labeling of cells and staining to view their morphology and variance</li> <li>• Worked with mouse whole blood, Human whole blood and 3 types of human leukemia cell lines</li> </ul> </p> <p><b>Dymach Pharma</b>, Vapi, India Jul 2015 – Aug 2015  QA Officer  <ul style="list-style-type: none"> <li>• Reviewed the Standard Operating Procedures and compared it to the standard template</li> <li>• Compared the values of the materials to their threshold value for quality check</li> <li>• Learned basic organization skills and how things work in a company</li> </ul> </p>
<b><u>PROJECTS:</u></b>	<p><b>Syracuse University</b>, Syracuse, NY Jan 2018 - present  In-vivo Evaluation of Low-frequency Ultrasound in Murine Leukemia  <ul style="list-style-type: none"> <li>• Achieved basic training in handling mice models for experimentation</li> <li>• Achieved training on IP, IV injections, blood collection and applying general anesthesia</li> <li>• Challenged the mice with leukemia and observed the effect of low- frequency ultrasound on it</li> </ul> </p> <p>Biomechanical Study of shoulder lateral abduction with resistance Sept 2016 – Dec 2016  <ul style="list-style-type: none"> <li>• Studied the biomechanics of muscles, tendons and cells related to shoulder abduction</li> <li>• Analyzed the variations in values with and without resistance through histograms</li> <li>• Lead the team to research about the standard values and analyzed it with the results obtained</li> </ul> </p>
<b><u>CERTIFICATIONS:</u></b>	<p>R programming certification from Udemy  MATLAB Certification from Udemy</p>
<b><u>ACTIVITIES:</u></b>	<p><b>Blog</b> Oct 2014 – Present  <ul style="list-style-type: none"> <li>• Messagethroughpoems.wordpress.com</li> </ul> </p>