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Ultrasonic blood fractionation: whole blood behaviour in an ultrasonic field

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Ultrasonic Blood Fractionation

Whole blood behaviour in an ultrasonic field

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A dissertation submitted to the Faculty of Engineering and the Built Environment,
University of the Witwatersrand, Johannesburg,
in fulfillment of the requirements for the degree of Master of Science in Engineering.

Johannesburg 2020

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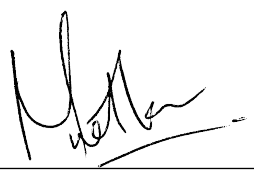
Declaration

I declare that this dissertation is my own, unaided work, except where otherwise acknowledged. It is being submitted for the degree of Master of Science in Engineering to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination to any other university.

Signed on 12 day of December 20 19



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SUPERVISOR: Prof D Rubin

SCHOOL/DEPARTMENT : Electrical and Information Engineering

DATE CONSIDERED: 05 July 2019

EXPIRY DATE: 05 July 2024

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Abstract

Fractionation of blood into its individual components is useful for diagnostic purposes. Ultrasound is a safe, non-invasive and affordable technology that can be implemented as an option for blood component fractionation, simply understood as component separation, based on the theory that mechanical sound fields can force cells to behave differently depending on its particular mechanical makeup. In this study, the proposed method of ultrasonic blood fractionation is investigated as an alternative to existing methods.

A specialised experimental set-up is designed and built to observe blood cell translation subjected to ultrasound.Red blood cell movement is more pronounced, moving in the opposite direction to white blood cells. White blood cells are only restricted in movement when trapped in a concentrated sample, not moving as much as when in a diluted sample. Erupted white blood cells move in a unique manner, different to intact white blood cells and red blood cells. Prolonged exposure to ultrasound results in white blood cell membrane expansion and ultimate eruption. The samples used were from blood banks thus the effects need to be repeated on fresh samples of blood for verification. It is concluded that exposing whole blood to ultrasound is capable of separating different components, however, more intense research in this field can verify the extent of its application as a separate technology that can compete in the industry.

This research forms a basis into the application of an alternative means to separate whole blood into separate components without damaging cells by using fundamental resonance properties of the cells as a means to separate the constituents. In addition, this research has establish groundwork necessary to be extended in a field relating to parasite-infested cell purification and further studies. The outcome of the experimental results can be used to explore if ultrasonic technology is viable in an extended application of separating healthy and parasitic blood cells, for example healthy and malaria-infested red blood cells, ex-vivo similar to a dialysis machine.

To...
My parents and sister

Acknowledgements

Funding from the University of the Witwatersrand, Johannesburg's School of Electrical Engineering, Biomedical Engineering Department Research fund (RINC).

Funding for the degree from Dr Raveendranathan's educational fund (MSCDEG 2018–2019, prot. 2018DAD).

Ethical clearance from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg (Wits, HREC Ref:R14/49, prot. M190346).

Ethical clearance from the Institution of Biosafety Committee of the University of the Witwatersrand, Johannesburg (Wits, IBC Ref:R14/49, prot. 20190601).

Ethical clearance and blood samples from the South African Blood Services NPC's Human Research Ethics Committee (SA NHREC Reg. REC-270606-013, prot. 2019/0468).

Dr Nel, for giving me the privilege to use the Mason Research Lab at the School of Surgery at hours convenient to my timetable. I would also like to thank Dr Nel for being the support in the medical field by showing the right path or introducing me to a fellow colleague when there were any biological walls I came to.

Prof Candy, Mr Moore and Ms Mazibuko, for providing assistance in the Mason Research Lab with necessary equipment, materials and storage facilities as well as going the extra mile and always coming up with alternate plans when roadblocks occurred.

Mr Classe, for providing the Olympus CKX31 microscope and supporting objectives to use for the experimental procedure. Mr Classe also provided technical support in helping obtain images and results optimal for analysis.

Dr Augustine and Ms Xulu, for providing the recipe for the Tyrodes Buffer.

Prof Crowther, for providing the chemical salts required for the Tyrodes Buffer from his lab and his wisdom in the medical field and the art of cricket.

Prof Postema and Prof Rubin, for being the great intellectual guides in this difficult path of academia. Thank you for the countless valuable lessons and care given through this journey as well as taking the time to show me the awe-inspiring avenue of research.

Mr Carlson, for stepping in and stepping up whenever necessary. For the adventurous ideas and brainstorming sessions as well as priceless guidance and his vast knowledge in the arts of academia.

Dr Keene, for helping me smooth out issues and pointing out some defining errors and plotholes I would not have picked up otherwise. The immense help and valuable lessons are greatly appreciated.

Ms Davids and Ms Adam, for always being the constant support system in my academic career as well as helping me swiftly tackle all the administrative roadblocks I had encountered. Thank you for

the multitude of conversations and constant reminders of tapping into my potential.

Mr Mofu, Dr Chingozha, Dr Genga, and Mr Vijayan, for allowing me to bounce off ideas and for providing the framework of support I needed even when I didn't ask for it. Thank you for going above and beyond to help me every time without even hesitating.

I'd like to specially thank Mr Mofu, Dr Chingozha and Mr Carlson for patiently reading through my document and for their detailed feedback that followed.

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List of Abbreviations

- BSC - Bubble-liposome Surrounded Cell
- CCD - Charged Couple Device
- CPD - Citrate–Phosphate–Dextrose
- DGC - Density Gradient Centrifugation
- EDTA - Ethylenediaminetetraacetic Acid
- FACS - Fluorescent Activated Cell Sorting
- HIFU - High Intensity Focused Ultrasound
- LCMD - Laser Capture Microdissection
- MI - Mechanical Index

Chapter 1

Introduction

Blood is the vital fluid within a body that influences the systemic functioning and well being, and as such an improved understanding of the properties of blood will guide in bettering therapy [1]. Studying the different components of blood assist in the defined understanding of the effect that each component has in the system. Efficient methods of whole blood fractionation, also expressed as whole blood separation, helps define quality studies and outcomes as the separated component is only tested.

Different blood components can be used to test for treatment of various illnesses and medical conditions without administering excess fluids to the patient, for example administering blood plasma without red blood cells decreases transfusion complications [2]. This form of treatment, named blood therapy, involves treating patients with a component of blood specific to their need [3]. In testing blood, the need to fractionate, or separate, blood components arises due to the blood cells and haemoglobin interfering with optical based techniques of measurements in analytical chemistry. Each component also requires specific tests that can only be applied to that component, therefore, the need for blood to be separated [4]. In the case of individually separated portions of diseased blood, the components are required to be pure and non-contaminated to safely deliver and administer the portion needed, thus requiring specialised separation processes.

Methods used to separate blood are based on a thorough understanding of the constituent components' physical or chemical properties so as to determine how to physically differentiate or what foreign chemicals to use to separate the components [3, 5]. Chemical methods are invasive and pose multiple threats to the cells and organs that the chemicals could travel to. Separating the constituents in a lab requires extensive bio-hazard and procedural training. The most common current method of component separation is using the physical characteristics of blood by centrifugation which relies on the sedimentation properties of the blood components. The separated parts, however, are adversely affected such that the properties of the cells change due to the high speeds in which the constituents are rotated in [5].

A proposed alternative means of separating blood into components without severely damaging the cells is by applying ultrasound, also known as ultrasound sonication. It uses the fundamental resonance properties associated to the different components to spread and fractionate, or separate, the constituents without causing damage to the cells. The research aims to find the acoustic regions that best separate the components due to its' inherent acoustic properties namely the frequency, pulse length and acoustic pressure range.

1.1 Research question

In what acoustic regime are blood components separated?

1.2 Research sub-questions

To answer the research question, the following objectives have been considered:

1. To determine the frequency range in which blood cells translate in an ultrasound field at clinically safe acoustic pressures.
2. To determine the pulse length range in which blood cells translate in an ultrasound field at clinically safe acoustic pressures.
3. To determine the acoustic pressure range in which blood cells translate in an ultrasound field at clinically safe acoustic pressures.

1.3 Dissertation content

Chapter 2 describes the background into the topic. The literature review provides information into the advancements of general and sonicated blood manipulation techniques. The readings will form the basis of the story-line for the dissertation giving analysis of the topic.

Chapter 3 is a technical description of the technology used to manipulate the blood samples in this study. The theoretical physics applied and the equipment and materials used are explained with supporting information to thoroughly understand the nature of the effects. The set-up and experimental procedure explains how to recreate the experiment to produce similar results for future testing purposes.

Chapter 4 contains the main results of the experiments. Results of preliminary and sample tests are presented to discuss optimal methods of experimentation. Section 4.3.1 presents the movement of blood cells, highlighting the clear distinction of white blood cells behaving differently to red blood cells. Section 4.3.3 presents findings of white blood cells more likely to erupt in sonicated fields. Section 4.3.2 presents findings of white blood cells expanding before eruption at rates faster in sonicated fields.

Chapter 5 discusses the outcomes, of the results represented in Chapter 4, and concluding remarks as well as valuable ideas of the applications and extensions of the work presented.

Chapter 2

Background and Literature review

Summary

This chapter presents the purpose of this research. The background provides the prerequisite concepts that aid in understanding the content of the topic. A general overview of the biology of blood is presented. The literature review discusses the current technology regarding ultrasonic blood manipulation and fractionation.

2.1 Background

2.1.1 Whole blood

Blood comprises of the liquid used to transport nutrients throughout the body, most importantly oxygen from the lungs to the rest of the body, within a circulatory system that also transports the waste products of metabolism to the excretory organs. It is mainly categorised as a tissue due to the interconnected nature of the mass, and is composed of various quantities of liquids and other formed elements. The idea that blood components can be used to cure specific blood-related diseases was practised fairly recently, however, blood-letting and transfusions had been widely practised for years [6]. After transfusing blood between different animal species, same animal species, and animals and humans, it was concluded that human blood should only be transfused to humans after a study originated from the need to address severe bleeding due to postpartum haemorrhaging [7].

Blood grouping and the Rhesus factor classifications resulted from comprehensive studies [6, 8, 9] identifying the various differences between the types of blood and thus the need to test for the similarities before administering the contents to a patient. The extensive studies prompted the need to store blood and form a service dedicated to the preservation and transportation of the tissue. This advancement was brought about with the aid of anticoagulants and blood banks committed to this need. Non-toxic compounds needed to be created to preserve and then later be administered to the patients, thus the advancement of the citrate–phosphate–dextrose (CPD) solution and other glycerol-based preservatives [10, 11, 12]. The length to which technology has advanced to incorporate storing and transfusing blood indicates its importance in modern medicine. The need to understand blood thus becomes of utmost importance.

Further advancement into the research of blood exhibited the precedence for certain components over others, thus prompting the technology into fractionation methods, for example how the need for blood plasma in the 1940s required separated parts of blood to extract blood plasma. The different components of blood treated different diseases and ailments without needing to administer any excess tissue and the process also avoided transfusion complications [6]. Each subdivided component of blood provided knowledge of the intricacies of the tissue thus examining and studying the tissue helps understand the vital tissue of the human body. Fractionation also allowed the separated constituents to be donated in parts where the unused components can then be preserved and administered differently, thus optimising on each donated amount of blood [3, 6].

Common methods of blood component separation are by centrifugation, membrane separation and chromatography. These methods incorporate sedimentation properties, laminar flow concepts and chemically induced separations respectively [6]. The main disadvantage of these methods is that they can change the physical properties of the cell. To combat the disadvantages of current methods, researchers have started studying the acoustic properties of blood as a basis for blood component separation using ultrasound technology.A study reported in [13] described that blood plasma's capacity to absorb ultrasound did not exclusively depend on the cells within blood, but could be due to the larger proteins within the plasma [13]. The concentration of albumin could also indicate a strong probability that these proteins bear responsibility in absorption of the signals. A study of the acoustic impedances of different animal tissues concluded that human tissue has an average acoustic impedance of 1.63×10^5 $\mathrm{g\,cm^{-2}\,s^{-1}}$. This average value was based on multiple patients being administered ultrasonic beams at different areas of the bodies [14].

The increasing research into the field of blood fractionation methods can aid in the field of blood therapy. The demand for blood within the population is also increasing [15], thus studying and consequently understanding the field of blood will open an avenue to better comprehend the vital tissue and how to effectively administer it.

2.1.2 Blood components

The different components of blood are summarised into Table 2.1 [16]:

Table 2.1: Components of blood.

Blood Component	Sub-component of blood	Details
Plasma	Water	Medium to carry all other organelles and proteins from various organs. This includes all the proteins, cells, chemicals, gasses and wastes.
	Main Proteins	Albumin - primarily controls the osmotic pressure within the vessels. These proteins also act as carriers of certain factors associated with the clotting process.
		Globulins - controls the osmotic pressure within vessels with albumin. Alpha and beta globulins serve as carriers and substrates for other proteins and substances. Gamma globulins form part of the immune response of the body for infections.
		Fibrinogen - involved in the clotting of blood.
	Other proteins	Most enzymes and hormones for regulatory processes are secreted directly into the plasma to be transported to the target organs.
	Other	Waste and other chemicals. This also includes a small fraction of lipids and gasses, for example phospholipids and nitrogen, as well as low concentrations of glucose and ions.
Cells	Erythrocytes (no nucleus)	Primary function is to transport oxygen from the lungs to the tissues and organs in exchange for carbon dioxide. The cells' diameter ranges from 7.5μm to 8.7μm and the thickness ranging from 1.7μm to 2.2μm. The cells have basic biconcave disk structure which is the standard form of the cell when at rest or not under external stress.
	Leukocytes (nucleus present)	Granulocytes - dominant type of white blood cell with a large number of granules and ranging from 12μm to 15μm in diameter. The cells are tasked with the process of assisting inflammations and attacking foreign chemicals by engulfing the bacteria or ingesting and processing antigens.
		Monocytes - primary role is to ingest and process the cells or infectious agents to be processed as foreign substances by the immune system. The cells are predominantly found in areas of inflammation or chronic infections and physical properties where the diameter of cells average from 15μm to 18μm with big indented/folded nuclei.
		Lymphocytes - part of the immune response system that move slowly due to the large nuclei that encompasses the entire cell. The diameter of the cells range from 7μm to 20μm. Most of the cells are concentrated in the lymph nodes and spleen, however, the cells when present in the circulatory system last from a few days to years.
	Platelets (Thrombocytes)	The smallest of the blood cells range from 2μm to 4μm in diameter. No nucleus present in the cell and look spheroid with hairlike filaments on the membrane. Primary function of the cells is to promote blood coagulation.

2.1.3 Movement of blood

Understanding the behaviour of the cells' movement without the influence of any external pressure sets the boundary conditions with which testing can be made. Despite variations in characteristics and morphology the basic cell structure comprises of a membrane that encloses a nucleus if present, cytoplasm and any other organelles. The membrane is the elastic semi-permeable outer surface that dictates the fluidity of the cell [17]. The flow of blood within a microvessel inside the body is determined by the osmotic pressure that in turn determines the capillary pressure. It is important to note that the flow of blood is slowest when travelling in a capillary allowing adequate time for metabolic processes. The concepts of haemodynamics can be extrapolated from the basic principles of fluid mechanics, however, the external changes to a vessel are induced from humoural and nervous factors [18]. Unlike red blood cells that do not deform unless there's external pressure, white blood cells in an active state deform spontaneously without external pressure. Passive state white blood cells require external pressure. The displacement of the white blood cell is linear to the pressure exerted [19]. Blood flow velocities are generally studied on large vessels such as the aorta or larger veins as the systemic methods of error are less prominent in large vessels. Cardiovascular systems work on a pump mechanism to transport the mass tissue around the body. Blood is also more viscous than water, however, it does not have uniform viscosity at all flow rates and becomes increasingly more non-Newtonian in micro-circulatory systems. These systems have more laminar flows as the Reynolds number of blood flow is low at values at around 1 and increase in larger systems like the aorta to a value of 4000 [20]. The general flow of blood within the systems is similar to that of a lumped mass travelling in a flexible tube that only expands to accommodate the travelling mass and returns to the natural state afterwards [20].

There stresses involved in the flow of the mass are due to wall shear stress in vessels, however, it varies from type of vessel as certain types of vessels are not necessarily circular to incorporate uniform stress. Another major stress on the mass of blood is the Hoop stress which occurs along the entire wall of the vessel that the mass travels in. This stress is the primary source of pressure that regulates the wall thickness and response to changes in blood pressure [21]. Unlike the larger vessels, smaller vessels have a more capillary-like action that dictates the flow of blood. The pressure differences that dictate the flow in larger cells do not apply to the smaller vessels. An important detail is that the flow of blood and the mechanisms of the system rely on the vessels and their mechanical properties which change from vessel to vessel, for example, the coronary artery has specialised features to accommodate the reverse in blood flow due to high pressured contractions [20]. Mechanical stresses induce changes in the vessels to accommodate these external changes in the form of quick internal bleeding and coagulation of tears, vessel dilations and contractions, vessels adapting to long term stresses. Ultimately corrective surgery such as stents or grafts are used when vessels can no longer naturally accommodate these changes [20].

Experiments on whole blood without lysing the red blood cells have an effect on the coagulation process that relies on thrombin or clotting factors to start the coagulation cascade which is a normal natural response of blood whereby cells migrate to form three dimensional clots. Conceptualising the formation of these clots, defined as thrombi, can be difficult as the fibrin mesh cannot be visualised easily. Thus the proteins and enzymes involved in the process do not need to be visualised or taken into consideration, however, the role of these micro-molecules needs to be understood. The coagulation process involves multiple cascading steps that involves these different molecules [22]. The process of coagulation opposes the flow of blood in a vessel and can obstruct components from moving along a

vessel, thus the initiation process of the clotting cascade needs to be delayed to allow a smooth flow of cells in an experimental procedure that focuses on component fractionation.

2.2 Literature review

2.2.1 Current methods of popular non-microfluidic blood fractionation

One of the earliest blood component fractionation, or separation, processes was the cold ethanol process by Cohn [3]. This five step method separates blood to the three main groups: albumin, immunoglobins and clotting factors [3, 23]. Over the course of the separation process, the pH of the solution needs to decrease from neutral, 7, to acidic, 0, and the temperature starts at room temperature to then change to below 0°C; whereas the concentrate of ethanol rises from 0% to 40% [24, 25]. The final product is a range of precipitates from each step. The effectiveness and aseptic nature of the by-products was grounds for the the popularity of the method. Dehydrating the proteins by the ethanol concentration forms the backbone of the process [25]. The effective method of using the Cohn process to isolate albumin resulted from the need to treat the Second World War patients due to the increased demand for the protein seeing its effectiveness in treating shock patients [24, 26]. The chemical process of separating the proteins is based on using the extensive knowledge of the different blood components' solubility properties. Another method used to separate proteins within the plasma is by using ammonium sulfate as salt in different concentrations for precipitation depending on the required proteins. Other chemical compounds used to separate blood include polyethylene glycol and lactate of 2-ethoxy-6,9-diaminoacridine [2]. The chemicals are added to the extracted blood to settle or raise the crystallised proteins and cells.

Plasma separation involves reducing whole blood to just the liquid medium that carries the formed cells. The advantage of using such a process is the reliability to test and compare for an elevated or decreased level of substances such as cholesterol, glucose and lactate to healthy levels within a blood stream. Blood component separation can also be performed using the components' physical characteristics. The main form of separation is by the process of centrifugation [27]. The process uses the sedimentation properties of whole blood by spinning a vial of blood at high speeds until the heavier cells settle to the bottom and the lighter cells form a layer between these heavy cells and a top layer of blood plasma. Centrifugation is mainly used for bulk processing situations or in conjunction with membrane filtration methods. More sophisticated and small scale processes, however, require methods that are intricately involved in the separation procedure to ensure higher purity and effectiveness. Density gradient centrifugation (DGC), initially developed for plant virus separation, separates matter based on the molecular masses depending on the density range. Fluorescent activated cell sorting (FACS) is a fast flow cytometry technology specialised to separate the suspension which is then induced into droplets and charged to be separated into different containers. The collected data of the feedback from the scatter or the fluorescence to further identify the cells individually via other methods such as flourochrome expression analysis or immunophenotyping. Laser capture microdissection (LCMD), microscopically isolates cells using a microscope to visualise areas that have to be cut away [27, 28]. The use of low gravitational pressure [29] and the intrinsic magnetic properties of cells [30] forms part of another method to separate the components using the physical characteristics. The use of the magnetic characteristics, however, needs the method to stimulate the magnetic properties of the cells

attaching paramagnetic materials to agglutination causing proteins or immunoglobulins by either making the components ingest the materials or chemically treating the blood cells to become magnetic [30]. Another fractionation method uses supplementary membranes and outlet pipes in a chamber using laminar flow designed to separate blood and segregates components as it flows through the chamber. The spinner chamber of the device incorporates the theory of inducing a magnetic instability within cells to be attracted such that they travel in a certain way [31].

All methods of fractionation require the separated components and the drawn blood to be optimally stored. The general rule of keeping the sample between 1 to 10°C is to preserve the functioning of the cells by slowing down the natural metabolic processes. Despite being exposed to room temperature for five hours, the quality of the red blood cells does not change enough for clinical significance [32, 33]. Room temperature exposure only increases adenosine triphosphate consumption indicating increased metabolic reactions and a decrease in the K^+ supernatant which indicates cell lesions when the level is raised. The decrease of the supernatant once again alludes to the increase in metabolic processes happening due to the increase in temperature [32, 33].

Ultrasound has been applied to multiple different cells to see if sonication or an acoustic field has an effect on these cells. The effect of ultrasound on donor blood at 2.25 MHz had been tested resulting in observing chromosomal abnormalities [34]. Platelet translation at 22 kHz avoids cell damage. Living-cell clusters appear in standing wave nodes for frequencies ranging between 1 – 3 MHz. Bacterial spores, endothelial cells, *E. coli* cells, and yeast cells all move to pressure nodes or anti-nodes in a standing acoustic wave field [34]. The peak-to-peak voltage of the applied field corresponds to the sound pressure, forcing the cells to be trapped in these due to primary radiation forces [34]. Red blood cells and particles with hydrophobic properties tended to nodes and anti-nodes in an acoustic field [35], where a study exploring acoustic standing waves within ring transducers [36] managed to cluster the red blood cells and like particles at fields of either 1 MHz or 3 MHz using a continuous-wave signal. This indicates that the cells travel to areas within a field of pressure differences and accumulate in these areas. Different behavioural aspects of cells in an acoustic field was presented in a study [37] that saw the clustering of Chinese hamster ovary cells compared to no clustering of human embryonic kidney cells exposed to the same conditions of a frequency at 7 MHz for 30s using a continuous-wave signal. Even in a sonicated sample containing both cells, only the ovary cells clustered.

In closing, the need to separate blood into different components is not only to be studied, but is also used to treat patients for targeted deficiencies. For example the component albumin is mainly used to treat shock, burns and loss of blood, IgG for immune deficiency disorders and prophylaxis, Factor VIII for haemophilia A, and Factor IX complex for haemophilia B as well as deficiencies of Factor II and Factor X [3].

2.2.2 Disadvantages of current methods of non-microfluidic blood fractionation

The cold ethanol process, though effective, is also expensive and poses health risks and discomfort for the professionals performing the process. The process is expensive and excessive as it produces more components of the blood than is necessary. Primarily aimed to produce albumin, the by-products of the process become wasteful as they are of less value in comparison to the main product. The multiple step-process consumes time and resources as albumin isolation is only achieved in the last step of the

process with less demand for the by-products [3].

The standard method of blood separation on a large scale is using a centrifuge, however, a minimum of 0.5ℓ of blood needs to be centrifuged at once [38], which is the limiting factor that sets the scope to bulk separation. The accelerated sedimentation process destroys blood cell membranes and residual cells still exist in the different layers. Centrifuging also limits accessibility of who can separate blood as it requires trained professionals to operate the device [31, 39]. The process is time consuming from requiring several minutes to hours to perform, however, the constituents still have a chance of not being properly separated if not carefully extracted [31]. Centrifuging also has limited particle separation control and focuses on mass separation of the components [38].

Membrane filtration is specific to the proteins and cells that need to be separated such that the membrane pores are catered only for those specified components. The specific nature of the separation process is practised in conjunction with chemical additives that need to crystallise or clear the blood concentration thus involving prior preparation. For example, filtration for lymphocytes required the sample to be treated with isotonic NH_4Cl to suspend the cells within the medium [40]. Chemical separation of any kind, such as the addition of ammonium sulfate, needs to be performed on already extracted blood. The blood is chemically changed thus the components that are not needed become wasted in the process of only acquiring the selected end-products [2].

DGC is another form of centrifugation that only separates the constituents, however, it does not address the damage in physical characteristics of the components. This method provides refined speed and precision of component separation as the only main advantages [27]. FACS depends on the interference of light and the addition of chemicals to highlight the specified components necessary to be separated. The cells require to be stained with antibodies thus the need to introduce new proteins into the sample [41]. Used in conjunction with flow cytometry, the high sensitivity of the technology requires for detection of certain and specific cells, thus the processing of blood into its separate components becomes time consuming [42]. LCMD is primarily used to harvest cells of interest by removing unwanted sets of cells. The aim of the technology is thus to procure pure cells from a population of interest [43]. Ferromagnetic detection of cells and separation thereof require for the intrinsic magnetic properties of the cells to be changed such that there is significant imbalance for the cells to be attracted externally. This involves introducing ferromagnetic chemicals or inducing such an imbalance which have lasting effects on the physical properties on the cells [30].

The disadvantages of the current methods put forth the need to investigate a method that can be used as a better alternative. The study described in [44], on the differentiation of microbubbles and free gas bubbles within liquid media, reaches into the potential that the different components of blood can have specific fundamental acoustic properties which can be used to differentiate each other. The advancements indicate the possibility of separation using ultrasound sonication and acoustic standing waves.

2.2.3 Ultrasound and ultrasonic transducers

Mechanical sound waves above 20 kHz are categorised as ultrasound due to these frequencies being above that of human hearing. The most common use of medical ultrasound is for soft tissue imag-

ing due to the minimal radiation risk associated with this technology. Additional advantages of this technology include how portable and inexpensive it is [45]. The typical range of medical ultrasonic frequencies are from 500 kHz to 30 MHz [46]. The waves propagate in the direction parallel to transmission, in essence longitudinal waves, along the straight axial direction, which is also referred to as the beam line along which the ultrasound propagates, unlike light waves which travel in the perpendicular direction, transverse waves in essence [44, 45].

Ultrasonic transducers predominantly use piezoelectricity to produce sound waves. This active material changes electrical energy to mechanical energy as pulses [47]. The piezoelectric layer of the transducer has two electrodes on either side. The outer later, after the electrode and closest to the load material, is the matching layer that is used to account for the acoustic impedance mismatch between the piezoelectric layer and the load [48]. A second layer on the other side of the transducer, after the electrode and on the opposite end of the load material, is the backing layer. This layer, behind the piezoelectric material is situated to reduce the ringing and excessive vibration. This prevents a reflected signal back to the piezoelectric layer, however, the layer's thickness compromises on the overall sensitivity of the device [48].

The device sound field consists of two sections, the near and far fields. The former is directly in front of the device consisting of acoustic differences of amplitudes due to wave interferences seeing that the wave propagation stems not from a point source, but rather the area of the piezoelectric device-layer. The near field has the distance of the transducer's natural focus and ends with its last maximum, the transition field to the far field, at the natural focus [47]. The far field starts after the end of the near field and is the gradual decrease of pressure which tends to zero and the beams are more uniform as the constructive and destructive interferences produce a uniform field. The waves are uniform and the field is also at maximum strength right after the transition field. The transducer not only produces waves, but also detects the feedback due to the reflections from the object it is produced for analysis. The diameter of a round-faced transducer, for example, is directly proportional to the near field distance where the waves are less uniform. The near field distance's non-uniform waves do not produce standing waves, and this distance is determined by the transducer radius. The reflected waves travel to the transducer's piezoelectric material, during pauses between pulses, which converts the mechanical waves to electronic signals [47].

Round-faced transducers, also referred to as piston-source transducers, do not maintain the produced beam in a cylindrical form as would have been expected. The beam tends to diverge outwards away from the axial line, creating a beam spread at an angle. The angle of divergence is calculated to the end of the spread from the axial line, thus half that of the beam spread. Ultrasonic waves can be sent in intervals as a pulsed signal or as a continuous wave. The former has time intervals in which bursts of pressure are exerted to the load medium, thus the frequency of these intervals can be changed. The pulses allow for enough time to receive the reflected signals. An important factor to consider is that the wavelength and frequency of ultrasound is inversely related, which in turn needs to be considered when deciding on the frequency of the pulses. Most importantly, medical therapeutic ultrasound classifies a continuous signal as thermal and a pulsed signal as non-thermal [49]. To control an adiabatic system, and to ensure the elasticity as well as other heat sensitive properties of cells are preserved, the pulsed waves are generally used.

Medical ultrasound is used to investigate soft tissues and organs within the body non-invasively. Immersing the transducer within a barrier gel-like media on the surface of the skin allows propagation of the waves to be optimal [50], where, the substance is used to form the transition for the sound waves and reduce friction when used externally [51]. An extended application of ultrasound is targeted therapy, as discussed in the following section.

2.2.4 Medical application of ultrasound

Medical ultrasound is involved in mainly imaging of fetus development [52] and detection of abnormal growths within bodies, specifically of cells and tumours [53]. Ultrasound technology is available, non invasive and its widespread use in the medical industry makes it affordable. The detecting of tumours based on the clustering properties of these masses is by monitoring the density of the cell cluster's growth. Ultrasound is used for imaging of the anatomy without the harmful effects of using an x-ray. This avenue of imaging provides a safer alternative where the ultrasound waves can penetrate deep enough within the body to produce details that can be used to supplement the doctor's diagnosis [53]. The application of ultrasonic sonication to tumour growth within mice aimed to determine if pulsed waves or a continuous wave had different effects on the overall growth of the tumour. Pulsed and low-power continuous sonication resulted in the same amount of total energy to the mice subjects [49]. The study aimed to provide an avenue to ultrasonic therapy as a means for cancers and tumour study within human bodies.

A device using ultrasonic frequencies has been patented to erode obstructive substances or matter from the blood vessels where the device is designed like a catheter device. It is used to deliver ultrasound from a source that will be external to the body of the subject via the contents of the catheter [54]. The device is thus a guiding mechanism to adapt the direction of the signal. Other applications of ultrasound in the field of medicine include localised heating of body tissues using short-wavelength pulses [13], This form of heat therapy is used to heal and relax tissues and to an extent heal bone fractures [55, 56]. These applications are based on the ability of sonication to induce thermal effects in the body. Localised therapy using ultrasound, named High Intensity Focused Ultrasound (HIFU), concentrate on areas that have surgical alternatives such as the brain, liver, prostate and the breast. If the surgical method is insufficient, ultrasound is used as an alternative to remove the diseased tissue by heating [57].

General application of ultrasound to living cells have been practised throughout the years where it promises an emerging field within the medical research and diagnostics avenue initiating from some of the earliest studies on Newt larvae to Madin-Darby canine kidney cells as the most recent [34]. Ultrasound is not only used for diagnostic therapy, but also for separating biological cells and manipulating these cells in liquid media using acoustic waves [34]. The movement of blood-like particles presents an avenue that can be explored to study the relationship of the particle's diameter to a peak frequency of an ultrasonic wave. A quantitative noninvasive analysis of the aggregation degree was done using mono-disperse acryl particles suspended in a thickener solution to imitate the behaviour of red blood cells [58]. A spectrum formed by a pulser oscillating transducer was attenuated by the particles. The wave reflected back onto a board where attenuation happens on the way back and is consequently captured and recorded. The principle reflection is of scattered waves when using these forms of particles. The findings established the higher the frequency, the diameter of the particle correspondingly is larger thus the coefficient of scattering is also stronger. The peak frequency of the reflected spectrum

reduces when plotted against particle size as a function. The oscillated pulsed signal is received in an attenuated form from the particles in suspension as attenuation caused by scattering and viscous based losses of signal strength [58].

A study reported in [59] manipulated bubbles in an acoustic field by trapping bubbles in nodes and varying the field to achieve this. This also ensures that the bubbles are not exposed to high pressure forces which would result in its destruction. The idea tackles the problem by introducing a form of targeted therapy as entering cells into a bloodstream risk a low concentration of them reaching the actual site. It is also observed that cells with a bubble attached to it are easier to control than a cell on its own. The cell density is reduced by attracting microbubbles onto the surface of these cells. This allows more controllability of the cells due to the microbubbles being more responsive to sonication. The final product is a specialised bubble-liposome surrounded cell (BSC). The aim of the study takes into account both the duration at which the sound can be applied and the extent to which they can be controlled when under the force as only one can be influenced over the other [59]. Cellular immunotherapy is an innovative new method that achieves targeted therapy by guiding the therapeutic cells to reach specific areas. A limitation of this technology is in the number of cells that can be guided. Bubbles aggregating to cells have been proposed to be less dense and succeeding in creating such a cluster, the study attached bubbles to Colon-26 cell surfaces [60]. The extension of the use of the Colon-26 cells expanded to incorporate CD8-positive T lymphocytes and BSCs which could be controlled more when the T-cells are surrounded by bubble-liposomes on the surface of the cells [60].

Destruction of bubbles in sonicated fields needs to be taken into account, thus needing an alternative method of controllability of the clusters. The six main phenomena of bubbles in sonicated fields are translation, fragmentation, coalescence, jetting clustering and cracking [61]. Coalescence amalgamates bubbles into one where cracking, unlike fragmentation that breaks a bubble into multiple smaller bubbles, is when the pressure difference between the bubble and outside are such that the shell of the bubble breaks after significant stretching to release a higher expansion amplitude bubble. The phenomenon of jetting is when the bubble forms a funnel-shaped jet protrusion that shoots liquid out [61]. The biological side-effects of ultrasound are utilised on bubbles and cells for these manipulations.

2.2.5 Ultrasound induced biological effects

Ultrasound affects the biology of the exposed tissue by producing thermal and mechanical changes. These two phenomena are represented as indices to analyse the effects of prolonged ultrasound exposure. The safety of this exposure needs to be regulated such that the effects produce no extended damaging consequences on the tissue. The following indices are not valid for contrast agents as the cavitaion threshold is lowered [62].

Mechanical Index

Inertial cavitation, the process of the violent collapse of a microbubble that is rapidly expanded due to high acoustic pressures, causes mechanical damage to the sample. The mechanical index (MI) is used to quantify this value and is expressed by the following equation [44, 62]

$$\mathrm{MI} = \frac{\mathrm{PNP}}{\sqrt{f_\mathrm{c}}}, \tag{2.1}$$

where PNP is the peak-negative pressure maximum value. This quantity is measured in water attenuated by a factor of 0.3 dB cm^{-1} MHz^{-1} due to the medium's attenuation coefficient. f_c is the centre frequency of the acoustic wave at a normalised frequency of 1 MHz. The MI provides an estimate of potential mechanical damage that could cause cavitation from the formation of these small microbubbles [44, 62]. Acoustic amplitudes are considered low at an MI of less than 0.3, however, at an MI ranging between the values of 0.3 to 0.7, it can cause possible damage to soft tissue. MI values above 0.7 increase risk of cavitation particularly in conjunction with ultrasound contrast agents, thus most sonication investigations are limited to MI values of 1.0 – 3.0 [44] and will be used as the limitations for this study as well. Noting that the approximate acoustic pressure of a sound waveform $P(t)$ can be extracted from the voltage reading using the equation below [63]

$$P(t) = \frac{V(t)}{M(f_{awf})}, \tag{2.2}$$

equation 2.2, measures the hydrophone voltage $V(t)$ and hydrophone sensitivity, $M(f_{awf})$, at the acoustic working frequency so as to take into account the varying sensitivity with respect to frequency as a function. This equation also applies to sources with narrow bands of sensitivity variation.

2.2.6 Microfluidic blood fraction using ultrasound

Microfluidics studies manipulation of sub-millimeter capillary fluid dynamics. The system adopts non-contact based cell separation that assists in using acoustic standing waves as a means to suspend particles in a medium to separate them. The particles will tend to move to the areas of different pressures in the medium based on the particle corresponding properties. The particle's inherent characteristics place it at certain points of the field as they separate based on the acoustic properties. The acoustic force will move them to either the node or an anti-node depending on the properties of the component [64].

Different sizes of polystyrene particles in a microfluidic field are used as a means to identify if ultrasonic fields are able to have an effect on the particles such that the behaviour of the particles are distinct to each other. Manual separation techniques have problems, this resulted in a means that differentiated cells using pressure waves to manipulate particles. Two walls form a transducer and and a corresponding reflection surface to create a standing wave field. The different approach refines the separation to a narrow band and upscales the process from previous studies through the use of lower frequencies. The particles move to different sections of the channel which then partition to an exit chamber. Suspended particles followed a straight line trajectory when the acoustic field is not energised, but moved in different directions once the acoustic field was turned on [65].

A micromachine controlled ultrasonic microfluidic separator uses inherent physical properties of particles to manipulate the suspended particles by maintaining a standing acoustic wave field within a cavity [66]. The aim of the study is to move particles within a flow, at a microscopic level, to an area so that it can be removed and separated. The concept is to clean the particles from a mixture using a micro-machine process that is custom built. The closed-loop controlled transducer creates a voltage-controlled oscillator to monitor and log the details of the frequency and voltage of the transducer as well as find an optimal point of operation to separate the particles [66].

A cavity needs to reflect an acoustic wave such that the spacing in the cavity and the roughness of the walls must be constant to support a standing wave. Radiation of high levels trap particles into clusters within channels affecting the laminar flow. These high radiation forces are created by high voltages (that also destroy the piezoelectric material of the transducer). In addition a flow rate reaches particle to positions in a standing field wave more easily than when in a stationary field. The applied field forces the particles into a flow that remains even after the sound field is turned off. An important note is that lower concentrations of blood in a sample mean the particles can be separated more efficiently due to the low saturation of cells in the channel [64]

A method developed in [39] separated lipid particles from erythrocytes using acoustic waves to separate the cells when suspended in a medium of bovine blood mixed with saline solution and triolein (a phospholipid-stabilised emulsion form). The particles separated in such a way that the red blood cells congregated towards the middle of the channel whereas the lipid particles were moving towards the walls of the channel. A triple channel outlet was used as mechanism to separate the cells into the two different outcomes. The study demonstrated the ability of the acoustic waves to discern the differences of the two cells indicating the possibility that it can be extended to more cells.

The concept of label-free separation of cells delves into the possibility of using intrinsic biomarkers as identifiers rather than additives that could change the properties of the components and particles in question. Marker examples include cell shape, electrical impedance, size etc. that will determine the choice of separation force that will need to be applied for the correct response. Microfluidic systems allow a single-cell based approach to manipulation [28]. Microfluidic separation methods separate components and particles in confined spaces, however, such an approach is not easy to implement in an in-vivo condition. This technology is thus a step in the direction of mass separation.

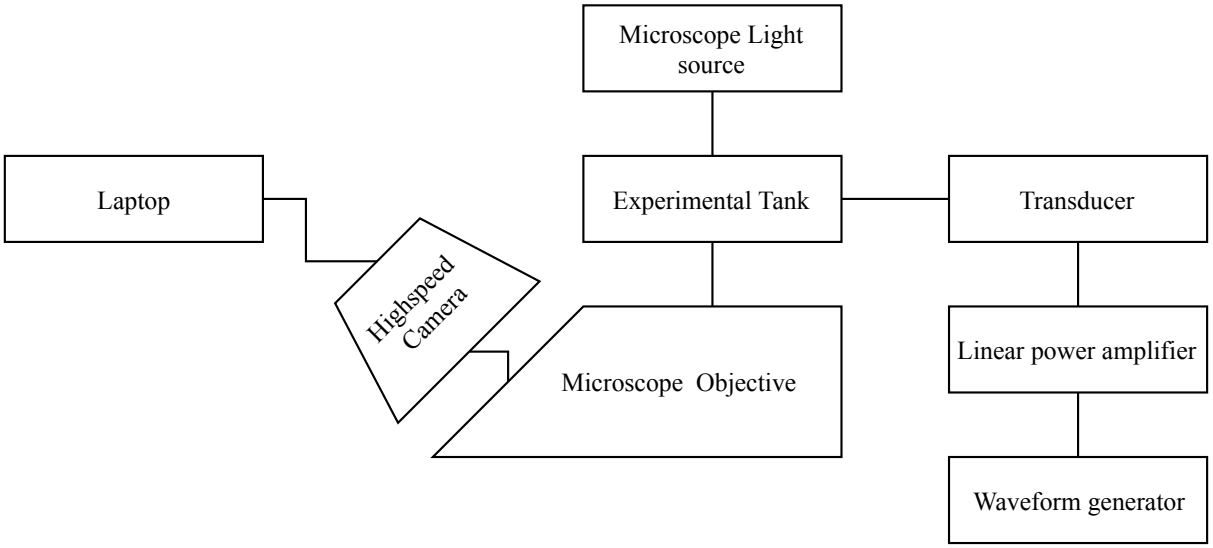
Chapter 3

Materials and Methods

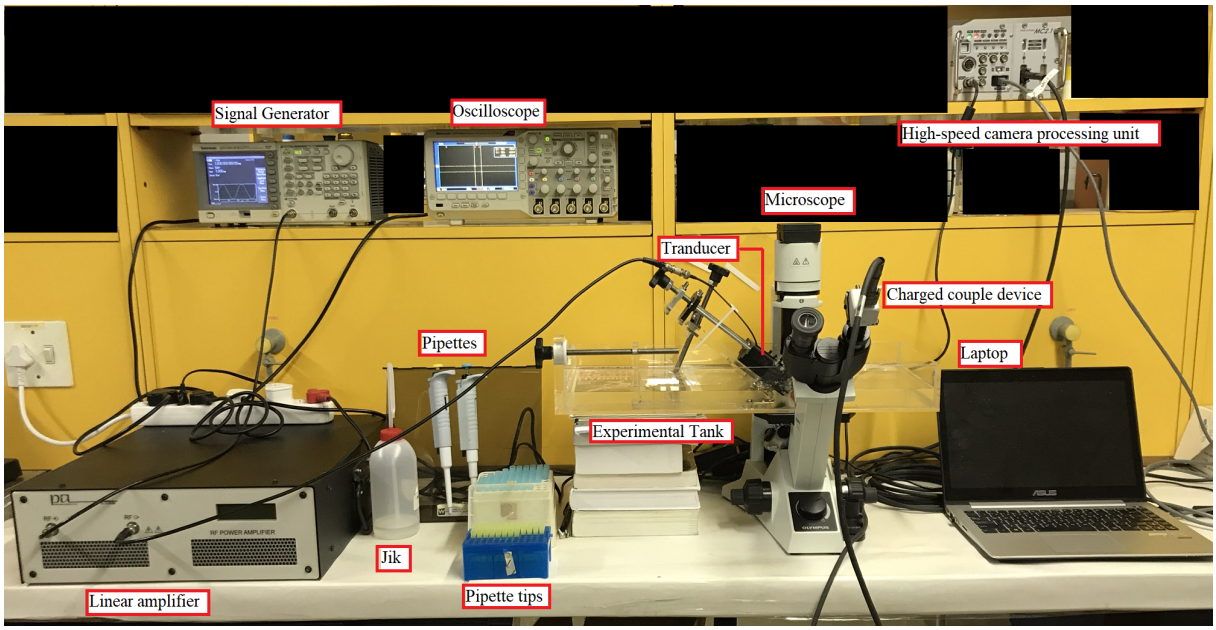
Summary

This chapter presents the guideline to the experimental procedure in this study. The information gathering techniques and the relevant equipment and materials used are also stated. The experimental set-up and the proposed working conditions of the set-up are illustrated in schematic diagrams and images to visualise the equipment and materials used. The recipe and method to prepare a solution that dilutes the sample is provided alongside the experimental procedure that details the steps involved in executing the full experiment.

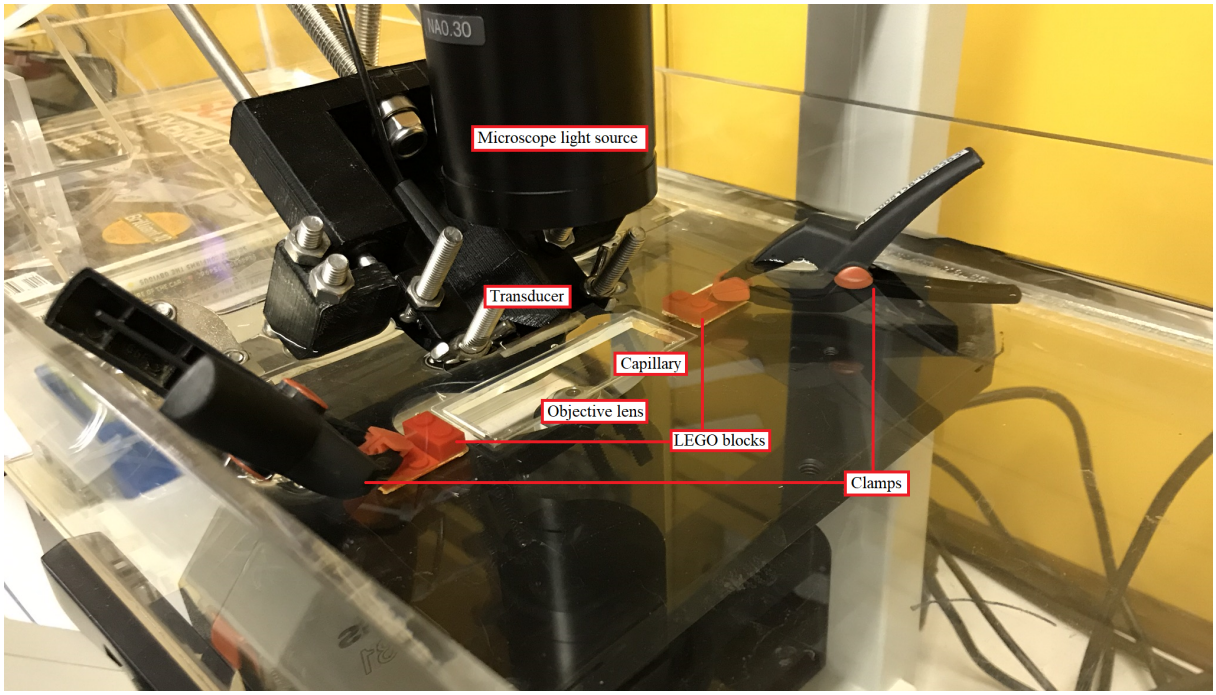
3.1 Experimental set-up



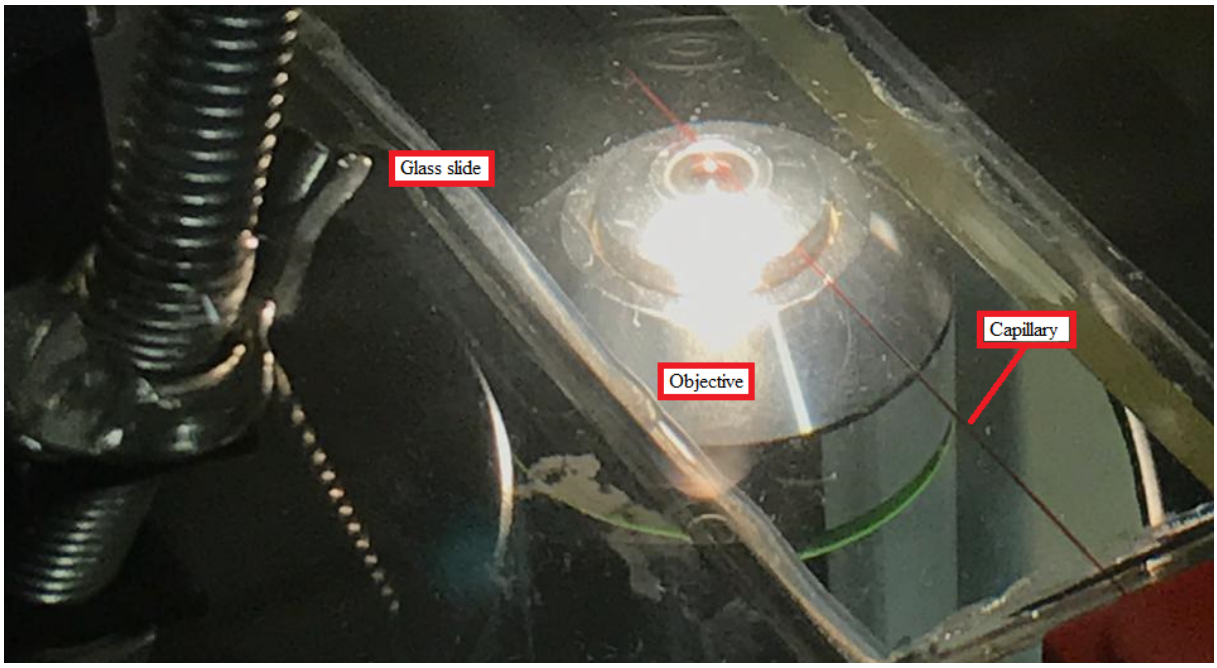
(a)


Signal Generator
Oscilloscope
High-speed camera processing unit
Microscope
Tranducer
Charged couple device
Pipettes
Laptop
Experimental Tank
Jik
Linear amplifier
Pipette tips

(b)


NA0.30
Microscope light source
Transducer
Capillary
Objective lens
LEGO blocks
Clamps

(c)



(d)

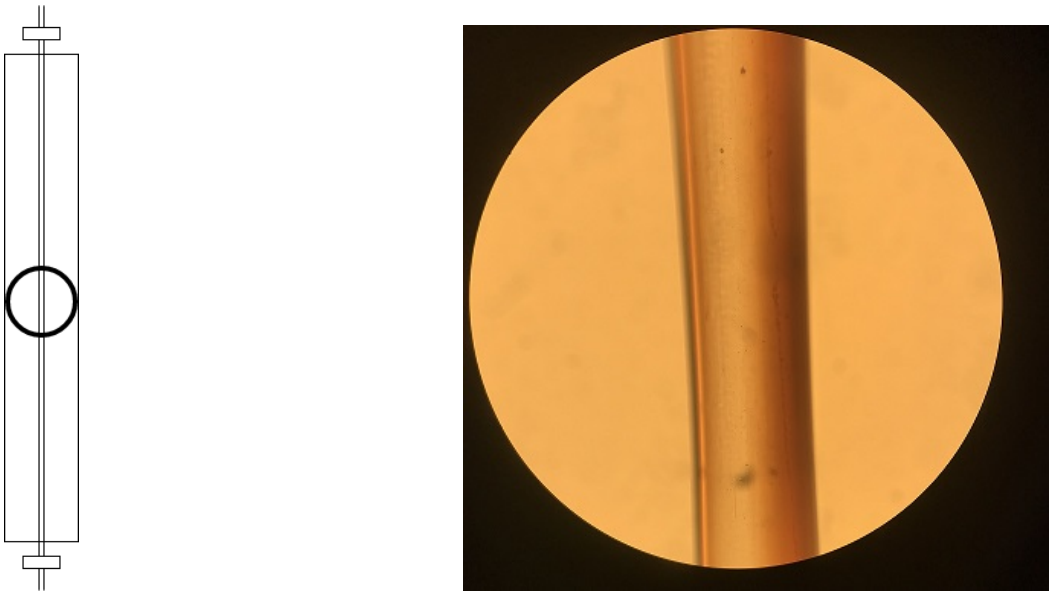
Figure 3.1: The final set-up used for the experimental procedures. Schematic line diagram (a) is of the experimental set-up, similar to [67].

The images in Figure 3.1 shows the set-up assembled for the experimental procedure. Figure 3.1a is a line drawing schematic overview of the set-up used for experimentation. The CKX31 inverted microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) has a 6V, 30W halogen-lamp light source positioned on the top which directs illumination into the Perspex experimental tank. The microscope's C-Plan N 20× objective lens (Olympus) below the tank has a working distance of 1.2 mm and a numerical aperture of 0.4. The $580 \times 235 \times 65$ $(\text{mm})^3$ tank contained degassed water as medium to allow maximum propagation of the sound waves and, to accommodate the inverted view, the tank opens on the bottom for a 26×76 $(\text{mm})^2$, and 1 mm thick, clear glass side that is fixed in place. The tank contains a Perspex plate which rests a transducer-holding bracket that can be adjusted. The transducer angle at which the transducer faces, and the distance from the glass opening, can be adjusted and secured in position.

A signal generated by the AFG 3021B arbitrary function generator (Tektronix Incorporated, Beaverton, Oregon, United States) was amplified by an A-150 55-dB linear power amplifier (ENI Technology, Inc., Rochester, NY) to drive the ultrasonic transducer within the experimental tank. The signals were pulses set at the centre frequency of the corresponding transducer. The transducer faced the sample material within the CUPROPHAN® RC55 cellulose capillary (Membrana GmbH, Wuppertal, Germany). The 14.5 cm capillary, with an inner diameter of 200 μm and wall thickness of 8 μm, was set in place on either side of the glass slide by LEGO® Part 3004 bricks (The LEGO Group, Billund, Denmark).

A FASTCAM MC1 high-speed camera's charge couple device (CCD) (Photron (Europe) Limited, West Wycombe, Bucks, United Kingdom) was connected to view from the objective lens of the microscope. The camera was fitted into the eye-piece with a custom designed C-mount adaptor 3D-printed for the objective. The camera unit was connected to a laptop to record all the videos and images.

Figure 3.1b corroborates the diagram of Figure 3.1a where the signal from the generator was amplified by the amplifier for the transducer. The set-up connected for commencement of the experimentation process has components mentioned in Figure 3.1a. The oscilloscope was connected to the hydrophone to collect the signal information to calculate corresponding MI values. The CCD, powered by the processing unit, fed the images into the laptop for data collection. 10 % diluted Jik^{TM} was used to clean the surfaces in case of spillages of blood. Pipettes and disposable pipette tips were used to transfer measured amounts of samples. Figure 3.1c is a close-up of inside the experimental tank of Figure 3.1b showing the path of the microscope light to the objective lens through the capillary. The LEGO® blocks, on the sides of the secured glass, and clamps secure the capillary in position. The clamps prevent the leakage of blood into the water by pressing the ends of the capillary and the LEGO® blocks minimise the movement of the capillary for effective sonication. The transducer was secured on the moveable Perspex plate in such a way that the face is directly in line with the capillary and the objective lens such that it was in the acoustic focus. A further close-up of the set-up illustrates the capillary placed directly above the 10× and 20× objective lens in Figure 3.1d. The secured capillary rests on the embedded clear glass slide which is placed so as to better view the sample in the capillary.

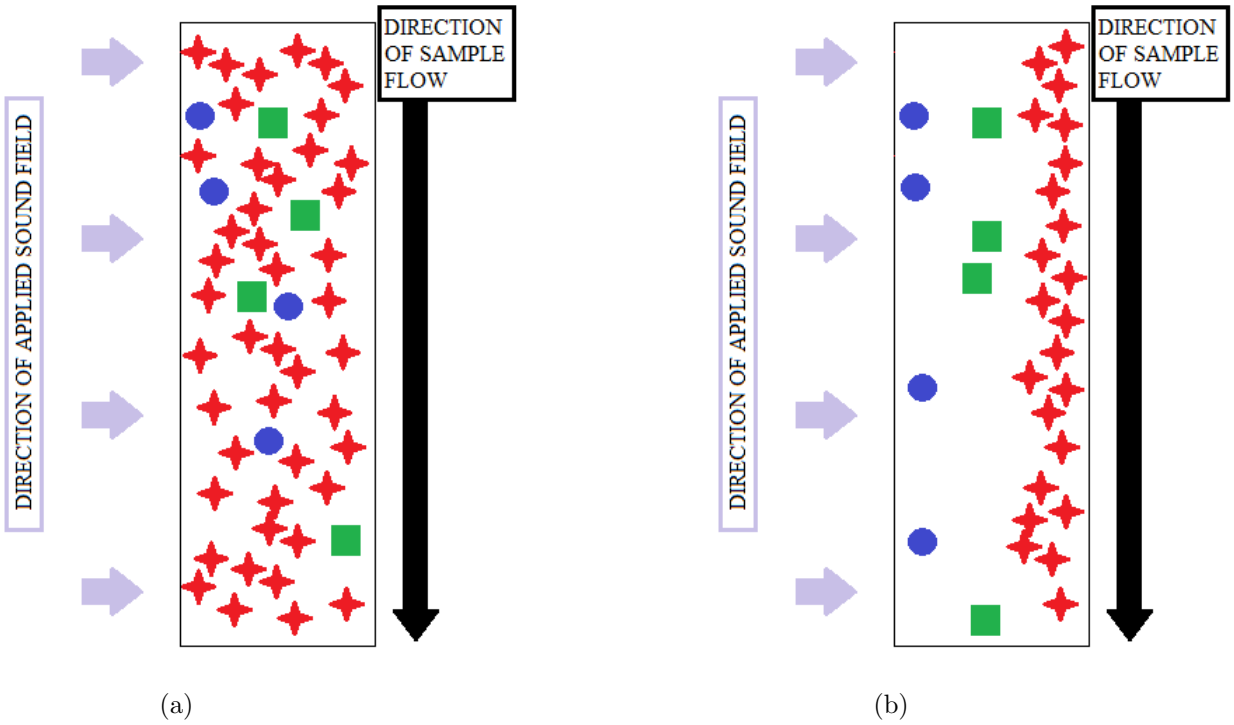


(a) (b)

Figure 3.2: Top view of the sample using the microscope explained with an illustration (a) and an corresponding view (b) through a 10× objective. The capillary size has an inner diameter of 200 μm and wall thickness of 8 μm.

Figure 3.2a illustrates the range of vision that was shown through the eye-piece. Two blocks on the top and bottom of the slide hold the capillary in place. The percentage of vision available to the

observer was restricted due to the objectives. The dark solid circle represents the area available from the objective. The set-up poses a restriction of view where the sample needs to be placed in such a way that the image being captured retains as much useful data as possible. Figure 3.2b shows the top view of the capillary through the eye-piece of the microscope at 10× magnification using an iPhone 7. The capillary needs to coincide with both the optical focus of the lens as well as the acoustic focus of the transducer.



(a) (b)

Figure 3.3: Proposed method of sonication which compares the sample before sonication (a) and after sonication (b). The purple arrows indicate the direction of the applied sound field and the black arrow pointing downwards indicate the movement of the sample's flow due to the natural streaming within the capillaries. Red crosses, blue circles and green squares represent different cell types possibly found in the sample.

Figure 3.3 depicts the manner in which a sound field applied is perpendicular to the flow of the sample. The capillary action induces the sample to stream within the confines of the area and the imposed sound field has an effect on the sample when moving the cells in a mechanical manner. Figure 3.3b proposes the result of sonication whereby the different cells of different properties rearrange to corresponding pressure points.

3.2 Transducer sound field calibration

Each transducer was tested in water using a hydrophone using the following set-up. The water was prepared and degassed to allow optimal wave propagation. The feedback of the hydrophone was converted into pressure from voltage to provide the conditions at which the transducer optimally performs at.

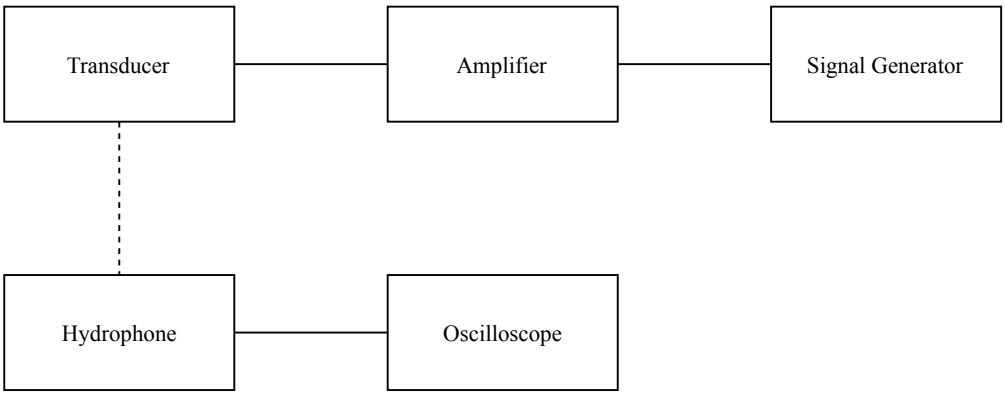


Figure 3.4: Schematic line diagram of the transducer testing set-up. Solid lines represent a direct connection. The dotted line represents indirect contact via reading signals using water as the medium.

The set-up procedure for the transducer was a standard method that can be easily repeated such that only a few parameters need to be changed when testing. The ultrasound field was positioned such that it coincides with the visual field. In Figure 3.4, the signal generator is the starting point of the set-up which was turned on to a set frequency, output voltage and number of cycles. The signal was then fed into an amplifier that is accordingly adjusted to provide an amplified signal to the transducer. To test and calibrate the acoustic field the HGL-0200 bullet hydrophone (Onda Corporation, Sunnyvale California, USA) connected into the AH-2010 pre-amplifier (Onda). The hydrophone was set in the field of the transducer to read the voltage equivalent of the pressure wave being emitted from the transducer which was displayed on the DPO 2014 digital phosphor oscilloscope (Tektronix). The oscilloscope was also used to read the signal being fed into the amplifier to ensure that there were no irregularities with the preset conditions, for example an internal setting that outputs a tenth of the amplitude despite indicating full output. The reading off the oscilloscope gave the amplified signal that was used to calculate the MI with equation 2.1. The pressure P(t) from the following equation [68]:

$$P(t) = \frac{V(t) \times A_{\text{relative}}}{G(f) \times M_{\text{c}}(f) \times \frac{\text{C}_\text{H}}{\text{C}_\text{H}+\text{C}_\text{A}}}, \tag{3.1}$$

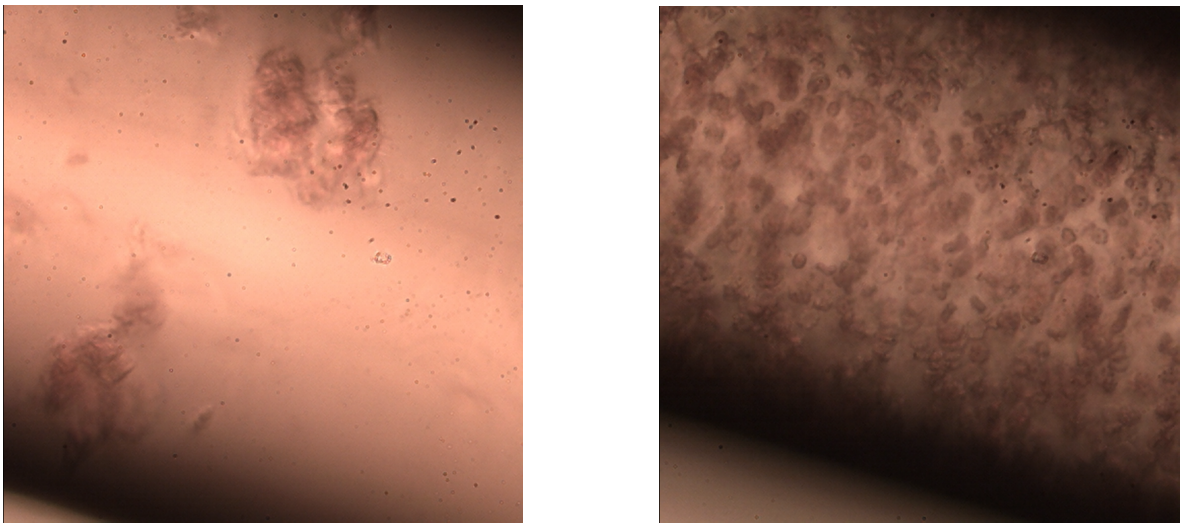
is a modified form of equation 2.2, incorporating extra components of accuracy namely A_{relative}, the component of relative amplitude with respect to the angle of the transducer on the hydrophone, G(f), the amplified gain of the signal as a function of frequency, $\text{M}_\text{c}(f)$, the electrical impedance dependant end-of-cable sensitivity of the detector, C_H, the capacitance of the hydrophone and C_A, the capacitance of the amplifier. The hydrophone's capacitive impedances have to be incorporated so as to account for the changes in value at different ranges. The components incorporated are from the hydrophone datasheet [68] and vary accordingly, hence the equation is not a standard and the static values are to be referenced from the datasheet. Hydrophone calibrations are usually magnitude only measurements without the addition of phase. The impact of phase and the average acoustic pressure experienced by the hydrophone affects the sensitivity component.

3.3 Field of view calibration

To calibrate the pixel area to the actual field being viewed, the calibration of the field of view is necessary. A 200 μm inner-diameter capillary was filled with Zuper Black tattoo ink (INTENSE Products, Inc., New Jersey, USA) to calibrate the frame in view of a 20× objective lens. Frames corresponding to 211 × 211 $(\mu m)^2$ area equate to the pixel size of 0.4 × 0.4 $(\mu m)^2$ area.
To confirm the method, Quantison^{TM} microbubbles (Upperton Ltd, Nottingham, UK) with a known diameter of 5 μm were used.

3.4 Tyrodes Buffer and anticoagulants

Sample dilution was necessary to observe the cells within the confines of the capillary. The concentration of an undiluted solution is unfit for sonication as there are too many red blood cells packed in the view, thus the need for using an adequate solution. Saline and distilled water are solutions unfit for the dilution of blood as saline dehydrated the cells into shells forcing water out due to the osmotic pressure change and the cells collapsing from the change of the cell's water content releasing into the solution. The dehydrated cells were observed to be much smaller and in shelled forms with little clotting. Distilled water forced the cells to fill up with water such that they formed spheres and popped cells. The result of this reaction contained clumped variations of cell remains from these cells floating in solution in large masses thus leaving no formed cells present in the capillary. The change in osmotic pressure from water resulted in popped cells as the water flowed into the cells such that it expanded beyond the change it could accommodate. Figure 3.5 illustrates the phenomena:



(a) (b)

Figure 3.5: View of the sample at 10/20× objective conveying the difference between a sample of blood diluted in distilled water (a) and saline solution (b).

Tyrodes Buffer ensures the solution that was mixed preserves not only the red blood cells and white blood cells but also platelets [69]. The following recipe was used: A salt mixture of 7.83g NaCl (EM-

SURE Merck, Darmstadt, West Germany), 0.1g $NaHCO_3$ (E Merck, Darmstadt, West Germany), 0.22g KCl (E Merck, Darmstadt, West Germany), 0.05g Na_2HPO_4 (E Merck, Darmstadt, West Germany), 0.1g $MgCl_2$ (E Merck, Darmstadt, West Germany) and 2.38g HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid) (Sigma-Aldrich, St. Louis, Mo, USA) was dissolved in 800 mℓ of distilled water using a magnetic mixer. The pH needs to be kept at 7.4, where HCl (E Merck, Darmstadt, West Germany) acid was used to change it. After mixing, 200 mℓ of distilled water was added to the solution. The bulk was stored at 4°C when not used. The solution was used to dilute the blood such that cells can be recognised when videoed.

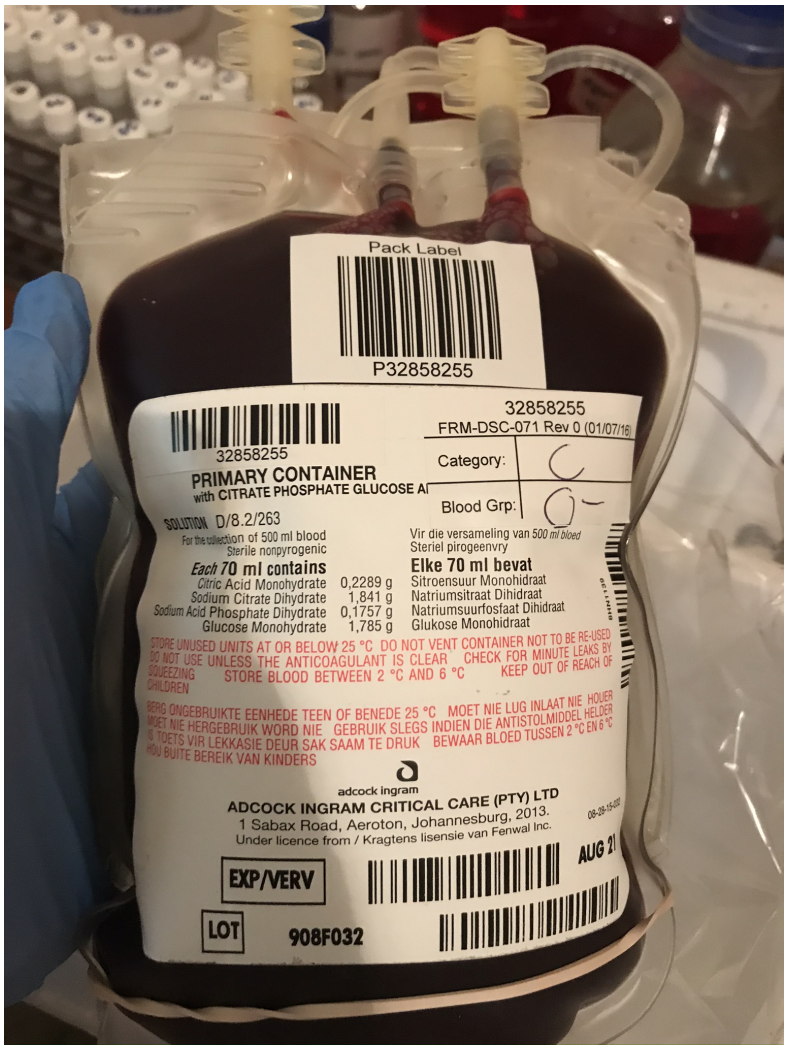


Figure 3.6: An example of the blood bag given from the SANBS. The description of the category and blood type are indicated on the top right of the bag. The anticoagulants are stated on the left side of the bag.

The primary container has citrate phosphate glucose as the anticoagulant. The other anticoagulants of the blood bag are citric acid monohydrate, sodium citrate dihyrate, sodium acid phosphate dihydrate and glucose monohydrate. The values of the contents are stated in Figure 3.6 for each 70 mℓ of blood.

Some of the delivered blood bags were of "Whole Blood Short Bleed" type, indicating that the bag was not filled and thus not ready for clinical transfusions. Such samples had large labels with the condition clearly stated on the front.

3.5 Experimental procedure

Blood from the blood-bag was drawn using a 50 mℓ syringe (Terumo (Philippines) Corporation, Binan, Laguna, Philippines) to transfer to vacutainers. The bulk draw was used to minimise the number of times the blood bag was punctured as the greater the chance the puncture hole is increased, the more air that enters the bag. Nichipet Ex pipettes (Nichiryo Co.,Ltd. Nishikata, Koshigaya-shi, Saitama Japan) gave measured samples for good ratios and calculation efficiencies. Two XC2 1-inch plastic spring clamps (Bessey Group, Bietigheim-Bissingen, Germany) which incorporates soft padding ensured the capillary was held in place such that when it was moved by the sound waves, it was secured on the tank. A standard procedure was followed to record results in a constant setting. 2 mℓ of Tyrodes Buffer, using a pipette, was added to a glass tube as mixing solution and 70 $\mu\ell$ of blood, using a pipette, from the vacutainer was transferred concurrently. The tube containing the solution was covered and agitated four times. A capillary was inserted into the tube where, allowing capillary action, three-quarters of the capillary was filled. To ensure this position, a mark was made on the capillary. The two sides of the capillary were clamped using the two LEGO® marks. The tank was moved into position such that the capillary was in view of the objective. After adjusting focus and lighting of the microscope, the camera was started to record the sample. The function generator set a quasi-continuous pulse with a width of 100 ms that was repeatedly aimed at the sample. The signals were set at the centre frequency of the transducer to allow maximum propagation of sound. The acoustic pulse length and pressure are both changed by modifying the amplitude of the signal and the number of cycles of the generated signal respectively. The amplitude of the signal was first measured during the calibration process using the hydrophone before the experiments were started. The number of cycles, also set during the calibration phase, corresponded to the on-time of the signal thus correlating to a pulse length. The frequency, pulse length range and pressure range are controlled variables that were modified between experimental sets. Once the procedure was started, the tank was set in place such that it was in optimal acoustic and observational focus such that the field of the transducer can be captured in the area of observation. After the tank was set in place, the tank was not moved. The capillary only needed to be placed such that it was in view again. Adjusting fine focus only needed to be accommodated when the experiments are being repeated. Figure 3.7 illustrates the procedure in a diagram.

The videos are then analysed to convert the data into cell trajectory information. Tracking of the cells using ImageJ's [70, 71] tracking plugin results in pixel coordinate values that correspond to coordinates of the cell in the field of view. Using 0 as the far left wall side of the capillary, the end of the 200 μm is the highest value pixel. The y-axis direction is not considered as the translation is of streaming and not that of sonication exclusively. The two-dimensional trajectory is thus an influence of both streaming and sonication which was not the pursued after data. Cell translation as a position is represented as a function of time and graphs are compiled using the MATLAB environment importing the .xlsx and .csv files of coordinate data which allows the use of the mathematical toolboxes to create the representations.

The procedure can be changed or manipulated to accommodate any different methods of testing such as with the preliminary tests. The tests incorporate minor changes in the preliminary phases to record

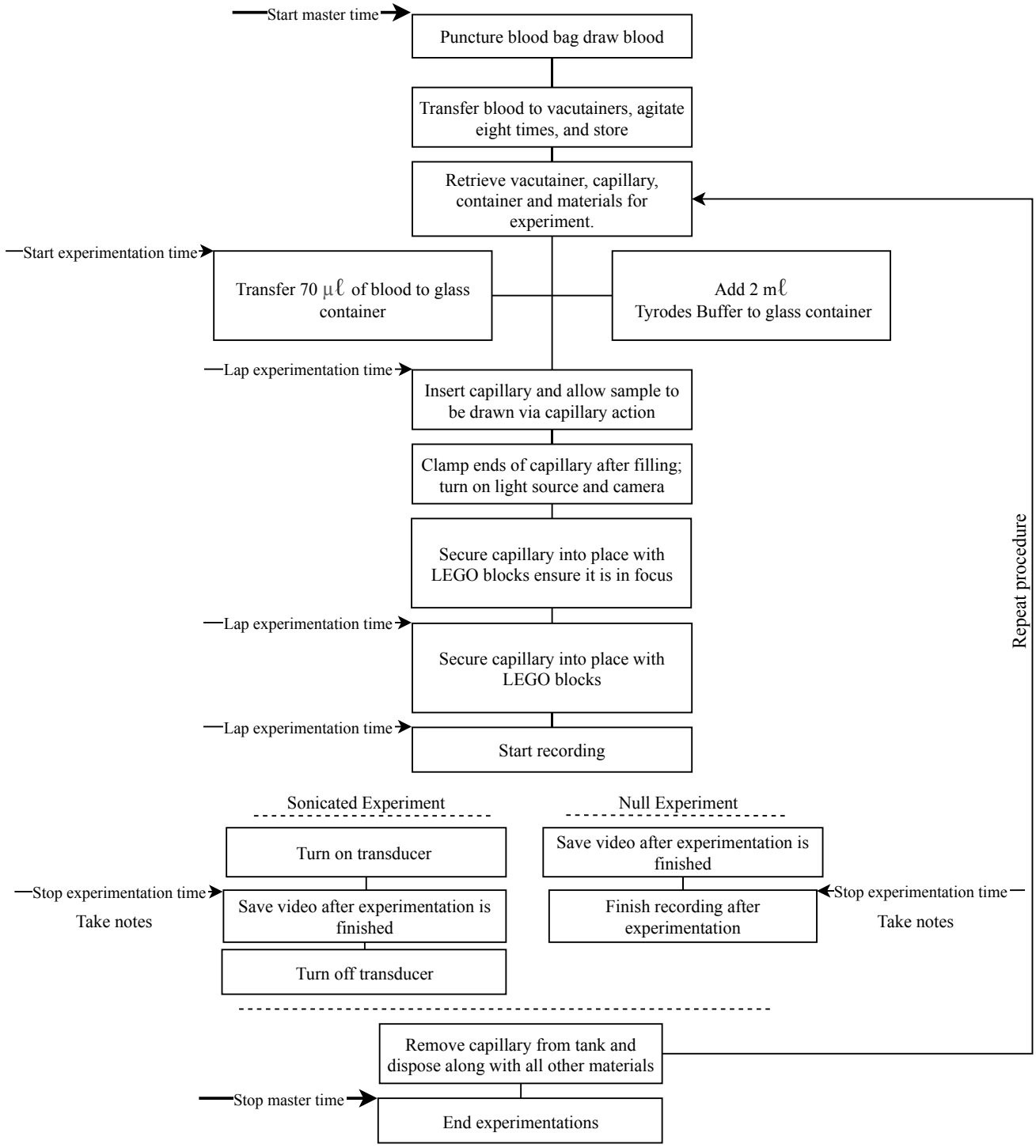


Figure 3.7: Schematic diagram of experimental procedure.

movement and find the optimal method of ameliorating blood damage. The procedure aims to ensure a well agitated sample of blood with enough Tyrodes Buffer such that the cells can be differentiated when videoed. The solution also dilutes the sample well enough such that it is less concentrated otherwise the sample cannot completely be filled in the capillary. This is due to the capillary's adhesion forces to the surface, due to the confined walls, that allow the liquid sample to fill the cavity of the tube, therefore, the adhesion forces overpower the cohesion forces between the liquid sample. This is unlike the concentrated sample where the cohesion forces are relatively stronger and travel less into the capillary. Figure 3.8 illustrates the three different capillary action test cases.

The testing procedures also identifies the best position of acoustic focus that can be captured using the microscope. Once in position, only the capillary needs to be placed and identified, by moving the set-up as little as possible.

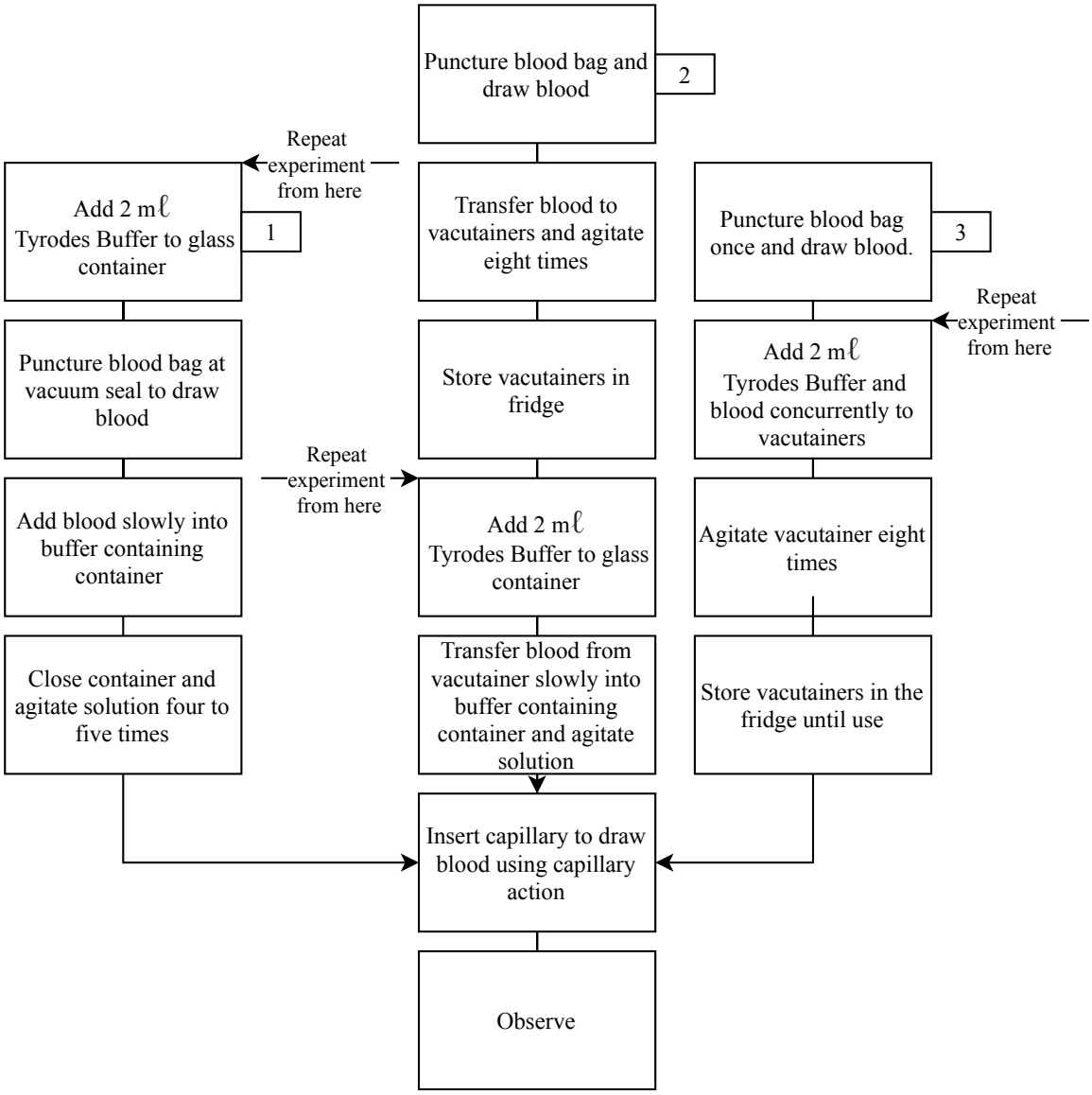


Figure 3.8: Schematic flow diagram of the three dilution testing methods for capillary action used to identify which method is optimal. Method 1 punctures the blood bag multiple times. Method 2 transfers blood into vacutainers. Method 3 transfers blood and Tyrodes Buffer to vacutainers. All three methods are tested by inserting a capillary to test the flow of the sample within the cavity.

Chapter 4

Results

Summary

This chapter presents the analysis of the experimental results and conclusions made in relation to the hypothesis stated in the research question. The chapter guides through the process of converting the acquired data to valuable information. Ethical clearance for protocol number M190346 was obtained from the University of the Witwatersrand's Human Ethics Committee and the required guidelines were strictly followed. The blood used was obtained from the South African National Blood Services that ensures thorough testing of substances unfit for transfusions, including infectious bacteria and viruses. Donated samples ensures the anonymity of the donors and are of Category C blood.

4.1 Transducer sound fields

The table below indicates tests done on three transducers available for the experimentation process. The table presents the output voltage values read from the transducer at different distances from the face of the transducer.

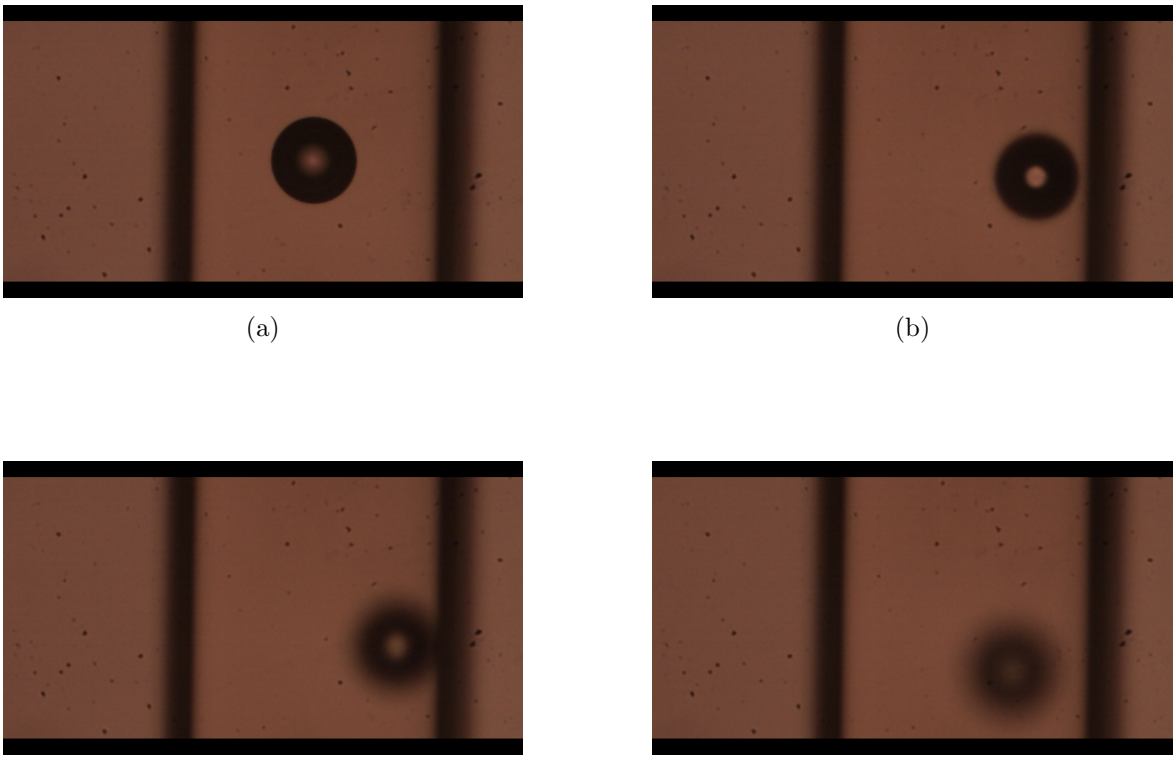
Table 4.1: Transducer testing results with 200 mV input voltage.

Transducer	Testing distance (cm)	Output amplitude (V)
14.286MHz with 28mm diameter	0	0.600
	5.7	0.140
	16	0.100
	25.5	0.065
5.681MHz with 28.1mm diameter	0	1.5
	5.7	0.400
	16	0.275
	25.5	0.162
285kHz with 18.85mm diameter	0	0.044
	5.7	0.04
	16	0.025
	25.5	0.02

4.2 Null testing

4.2.1 Microbubbles within an acoustic field

A microbubble within an acoustic field was captured using the high-speed camera. Microbubbles are very susceptible to ultrasound and move at very high speeds when in an acoustic field needing a high-speed camera to properly track their movements [61].



(a) (b)

(c) (d)

Figure 4.1: Top view of the movement of a bubble within a 200 μm inner-diameter capillary after applying a 200 kHz transducer to the bubble. The bubble initially moves from the top of the capillary to the middle, as well as a circular motion from the middle, to the right side of the capillary and then back closer to the middle again. The movement occurred over the span of 22 ms and captured at 10/20× objective.

Initial condition of the bubble prior to any form of sonication applied to the bubble is presented in Figure 4.1a. Approximately 6 ms later, in Figure4.1b, the bubble has moved to the right side of the capillary. The blurrier image indicates that the bubble has moved a little away from the focus. Approximately 10 ms after initial position the image, Figure 4.1c, becomes blurrier indicating that the bubble is moving further away from focus in the capillary. Approximately 22 ms after initial position, the bubble moves to the right and is in the middle of the capillary again, however, it is still away from

the initial focus thus indicating that the bubble has moved in the capillary. It took approximately 10 ms for the bubble to move to the middle-left and come to a stop in Figure 4.1d.

4.2.2 Whole blood coagulation in a testing well

Null testing of sample blood used to identify the mannerisms of the clotting process with and without an external stimulant is tested to see the expected outcome of the general behaviour of the cells. Whole blood with a 3.2% citrate concentration is used as the sample and different amounts of thrombin are used to see if there is a visible change of the coagulation process. The change in amount of thrombin only has an effect on the speed of the process.

The sample is pipetted into a well, thereafter a volume of thrombin is added and then the sample is agitated by hand to produce a reaction. The test is used as an investigative standard to hold against as the natural baseline for future experiments using thrombin and ultrasound. The formation of clots and the rouleaux formation only speed up in the presence of thrombin. The change in thrombin volume only has an effect on the speed at which the coagulation process occurs resulting in stacking and other reactions. The rate at which the thrombin volumes have an effect are inferred from the brightness of the light coming through the sample. Samples with lower volumes of thrombin pass more light indicating that the clots have not been fully formed. The use of the mechanical agitator is decided against as the irregular shaking results in fragmented clots and the rouleaux formation is no longer visible. The samples are thus gently agitated by hand in circles. The citrated blood ensures delayed coagulation resulting in cells spread out and stacking on top of each other at a slow pace. The cells stack in no particular order. Multiple observations through the microscope did not show a uniform method in how the cells gravitated towards each other.

The stacking of the blood cells formulated an issue that needs to be addressed as the three-dimensional image blurs the cells on the different planes making it difficult to discern the cells. This problematic view cannot be changed as smearing the blood on a glass slide will hinder with the natural clotting process and allowing the clot to form distorts the amount of data captured as the light source travelling through the clots become weaker as the clots become more dense. These poorly recorded results cannot be used for measurements thus relying on manual visual observation.

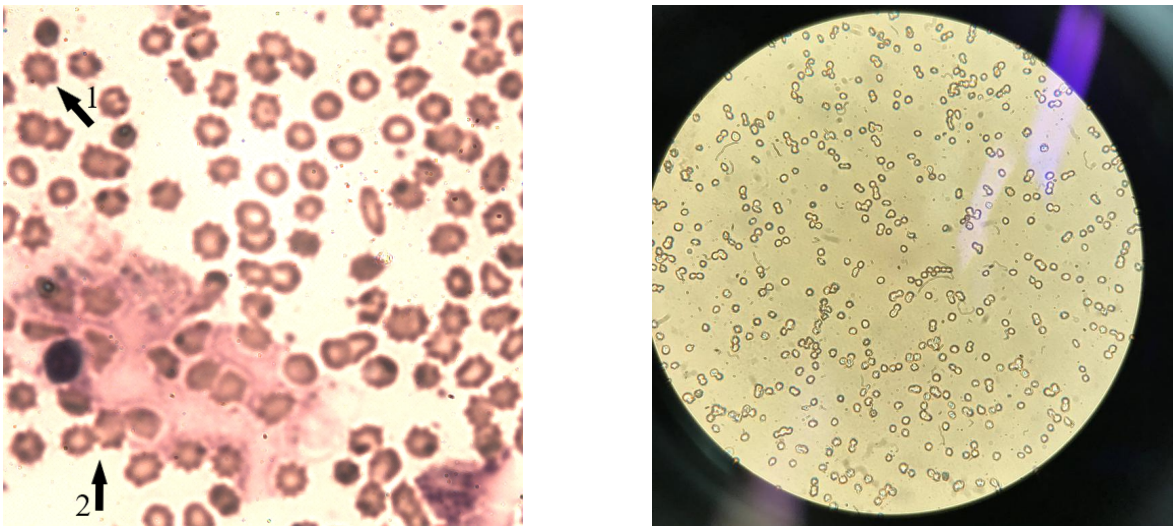
4.2.3 Diluted blood sample smears

Blood was first smeared onto a slide and cover slip to see how dense and agitated it is. This was due to the uncertainty of how much the sample had been damaged from being transferred to a vacutainer tube. The vacuette vacutainer tubes contains a sodium citrate anti-coagulant solution of 0.109 mol/l (3.2%) translating to one part citrate for every nine parts blood. The anticoagulant aims to slow down clotting of the cells such that it can be studied under the microscope. The citrate anticoagulant is prefered as the the strict composition of the ethylenediaminetetraacetic acid (EDTA) containing tubes means that the calcification of the natural chemicals in the blood are severely compromised further damaging the platelets and soft-membrane cells left from the blood bags.

Initial experiments were drops of blood on a slide and set into place using a cover slip. Certain layers of cells streamed across the field of view, however, the bottom most and top most layers closest to the slide and cover slip contained cells stuck from moving around. The cells on the slide streamed across

the field of view due to it being smeared and consequently heated by the light of the microscope or the general capillary action within the thin space.

Blood samples initially inserted into a capillary populated the space such that the sample needs to be diluted for sufficient space between cells. This resulted in a mock-test to see if cells can be diluted in distilled water or saline solution of 0.09%. Two standard test samples contained 70 μl of blood with 5ml of diluted water in one tube and 5ml of saline in another tube. The samples observed under the high-speed camera shows dehydrated cells when in a saline solution and clumped cells in the water solution. The Tyrodes Buffer neither clumped nor dehydrated the cells.



(a) (b)

Figure 4.2: Stained blood smears showing distribution of the type of cells typically found in a sample.

Figure 4.2a is a smeared sample using the high-speed camera. Arrow 1 shows spiked spherical acanthocytes. Arrow 2 shows a cluster of popped white blood cells conglomerated into one large mass. Figure 4.2b is of the same spread of cells through the microscope with a 20× objective taken using an iPhone 7. Unseen on the smear are the presence of platelets which were recorded in other smears.

4.2.4 Diluted whole blood capillary-action testing

An hourly experiment conducted over a span of 10 hours set to determine the best method of storing blood to ensure the blood remains with as little change as possible. The samples were recorded and analysed to see if there were any significant changes in composition of the cells or major discolouration which can indicate the lysing of red blood cells. Three test methods consisted of determining the best solution to preserving the quality of the blood. The first test involved testing blood straight out the blood bag and diluting it in Tyrodes Buffer before inserting it into a capillary. The second test premixed the Tyrodes Buffer with the blood and then stored it in the vacutainer. The sample was then inserted into the capillary. The third test stored blood in vacutainers and then mixed with Tyrodes Buffer before inserting it into the capillary. The result was a success only if the sample was able to be

inserted in the capillary through capillary action without being stuck mid-way. The consistency of the dilution determined this factor and the method that takes the least time was chosen.

Removing blood straight from the blood bag poses the risk of exposure to the air and oxygen regardless of the vacuum seal. The hollow needle used to prick the seal kept opening the bag such that the hole got bigger, exposing more and more of the blood to the air. Over time the blood in the blood bag started separating into various layers, indicating that the sedimentation process was taking place. The blood being inserted into the capillary also started getting too thick to be pulled all the way into the cavity as there was no coagulation agent like that of the citrate in vacutainers which further aided in slowing down the coagulation cascade. After the first few hours, due to the condition of the blood bag, this method of testing was deemed no longer fit to be continued.

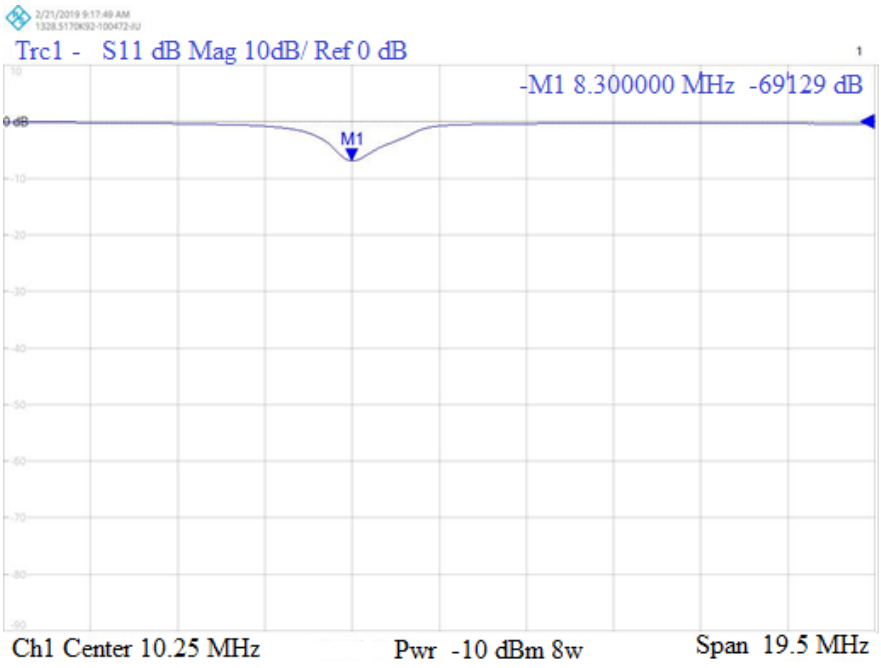
Premixing the solution required filling a large syringe with Tyrodes Buffer and then filling the remainder of the syringe with blood. This mix was then transferred into a set number of vacutainers that held the solution over the hours. The solution not only sedimented over time but the capillary action slowed considerably as time progressed. The citrate in the vacutainers seemed to no longer have an effect of slowing as the cells started sedimenting in the vacutainers. Additional agitation loosened up the sedimented cells, however, extra dilution of Tyrodes Buffer was necessary to see the cells clearly in the capillary.

Storing the blood straight from the bag into a vacutainer allowed the sample to mix with the citrate and further slow the coagulating. The vacutainer removed from the cool storage was opened and the sample was then diluted with Tyrodes Buffer before it was inserted into the capillary. With this method cells were observed to flow steadily in the capillary and the samples also seemed not to change over time. A red discolouration in the plasma indicated haemolysis, and this method observed no discoloured solution over time. This was further established after studying the stained smears of the sample over time. The citrate kept the blood from sedimenting and only showed these signs of sedimentation after being stored for 8 hours. For the remainder of the experimental procedure this method was adopted.

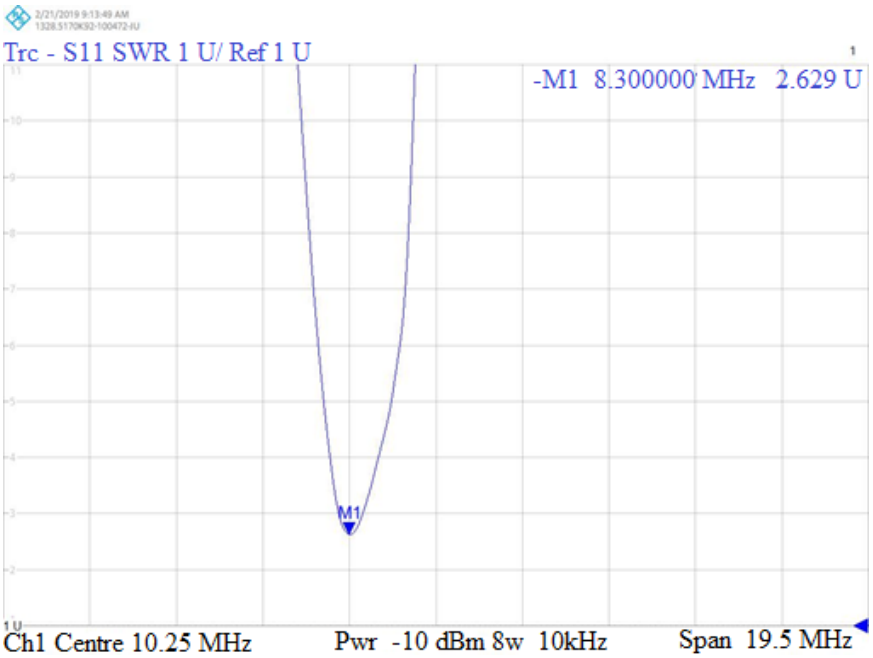
The best method of capillary action testing is using coloured water to follow the distance that the liquid travels.

4.3 Sonicated testing

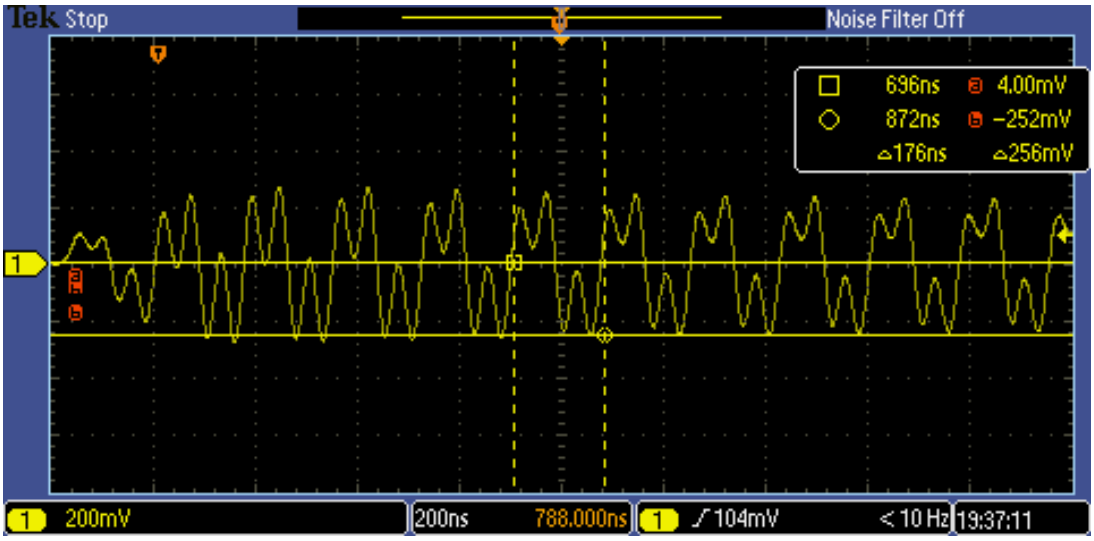
All sonicated tests used the 5.681 MHz transducer. The resultant period of the output wave steadied at 176 ns, indicating the actual output frequency of the transducer to be 5.681 MHz. The amplitude, the frequency, the number of cycles, the angle and the distance between the transducer and the capillary played a role in changing the MI and behaviour of the cells when exposed to sonication. This was the only transducer that resulted in visible change to the cells despite having a total of three transducers, thus the use of the other two transducers were regarded as not viable for the purpose of cell translation. The sonicated experiments ranged from an MI of 0.2 to 0.4.


Trc1 - S11 dB Mag 10dB/ Ref 0 dB
-M1 8.300000 MHz -69.129 dB
M1
Ch1 Center 10.25 MHz
Pwr -10 dBm 8w
Span 19.5 MHz

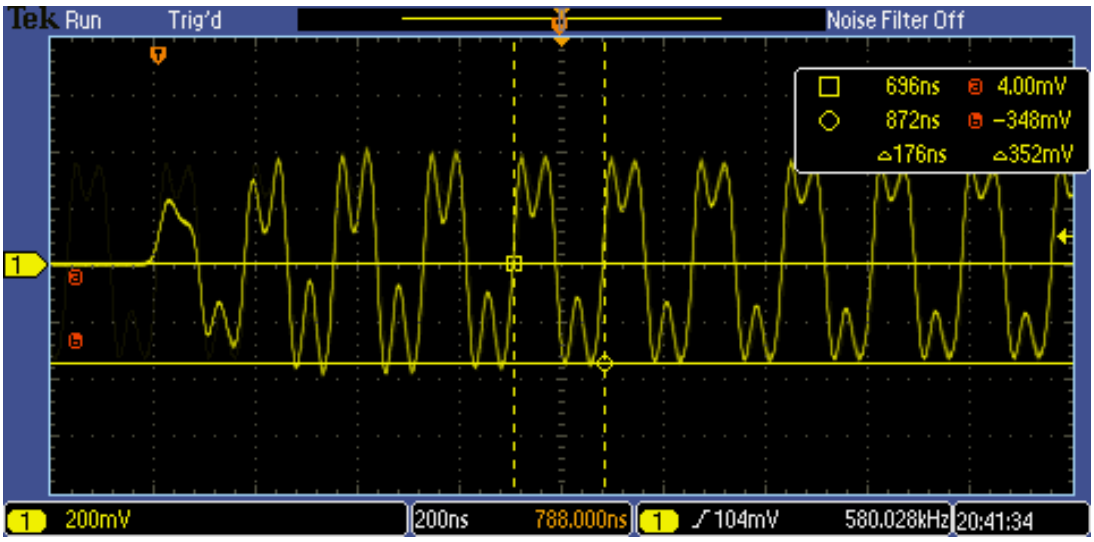
(a)


Trc - S11 SWR 1 U/ Ref 1 U
-M1 8.300000 MHz 2.629 U
M1
Ch1 Centre 10.25 MHz
Pwr -10 dBm 8w 10kHz
Span 19.5 MHz

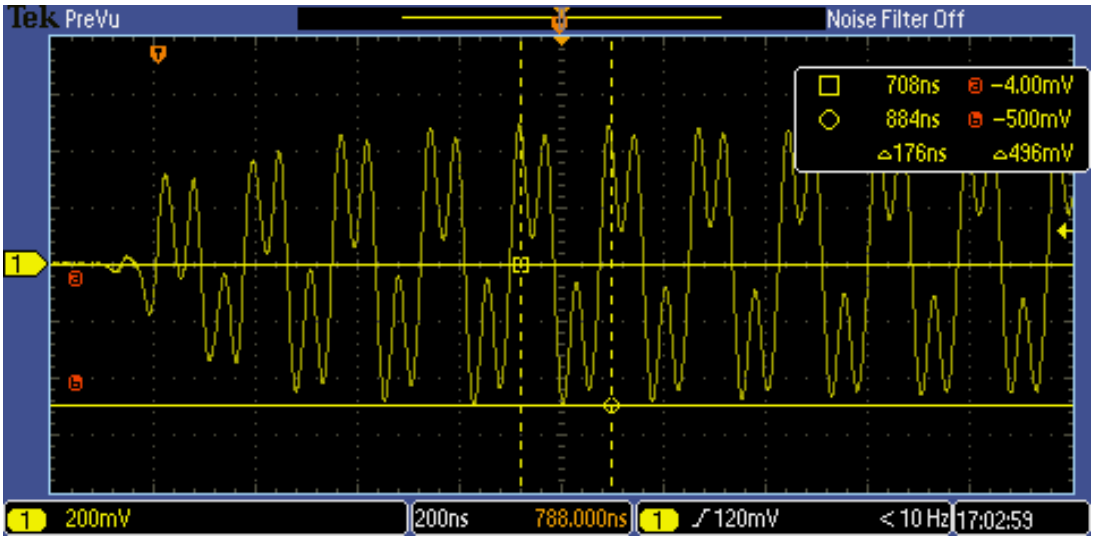
(b)


Tek Stop
Noise Filter Off
696ns 4.00mV
872ns −252mV
Δ176ns Δ256mV
1 200mV
200ns 788.000ns
1 ∕ 104mV
< 10 Hz
19:37:11

(c)


Tek Run Trig'd
Noise Filter Off
696ns 4.00mV
872ns −348mV
Δ176ns Δ352mV
1 200mV
200ns 788.000ns
1 ∕ 104mV
580.028kHz
20:41:34

(d)

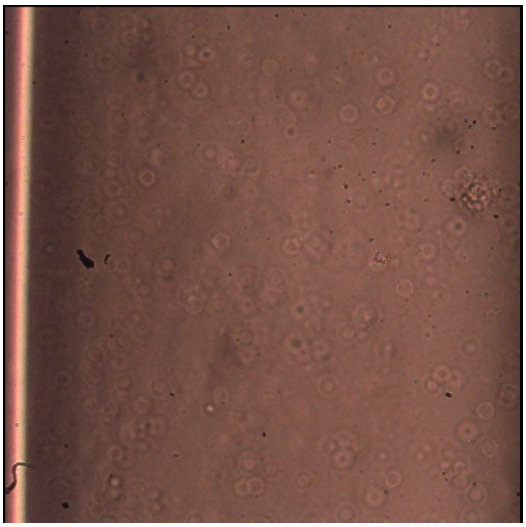


(e)

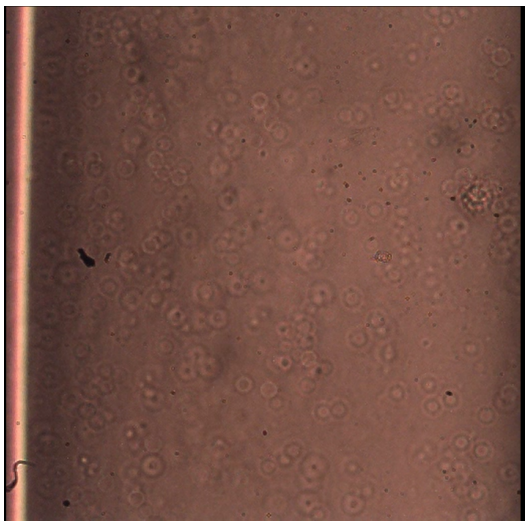
Figure 4.3: The dB-magnitude curve (a), standing wave ratio curve (b) and the examples of waves recorded on the oscilloscope yielding waves 256 mV, 352 mV and 496 mV (d-e).

4.3.1 Movement

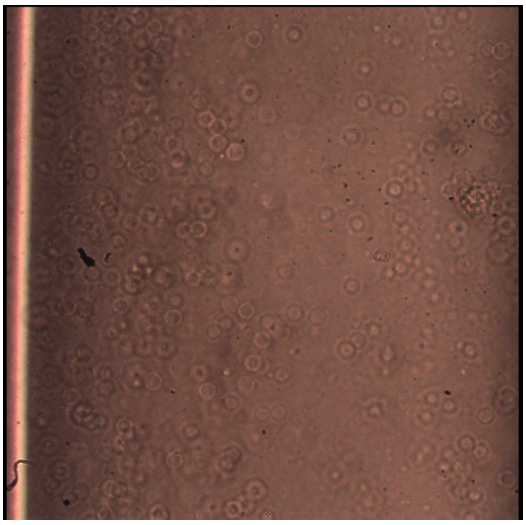
Clear movement of cells were captured when the clearly defined cells are traced using the ImageJ platform to track the trajectory travelled by the cells from frame to frame. Figure 4.4 shows the movement of red blood cells in the capillary.



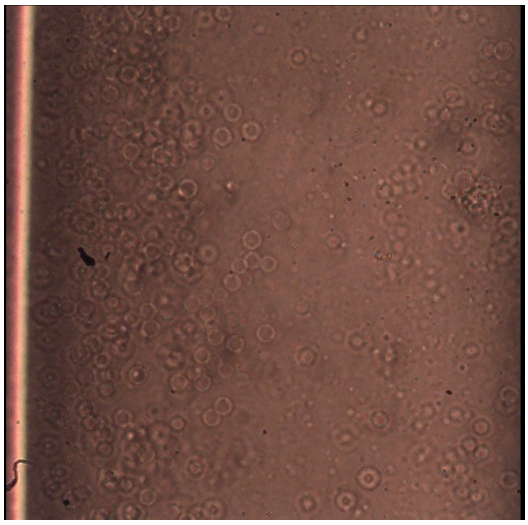
(a)



(b)



(c)

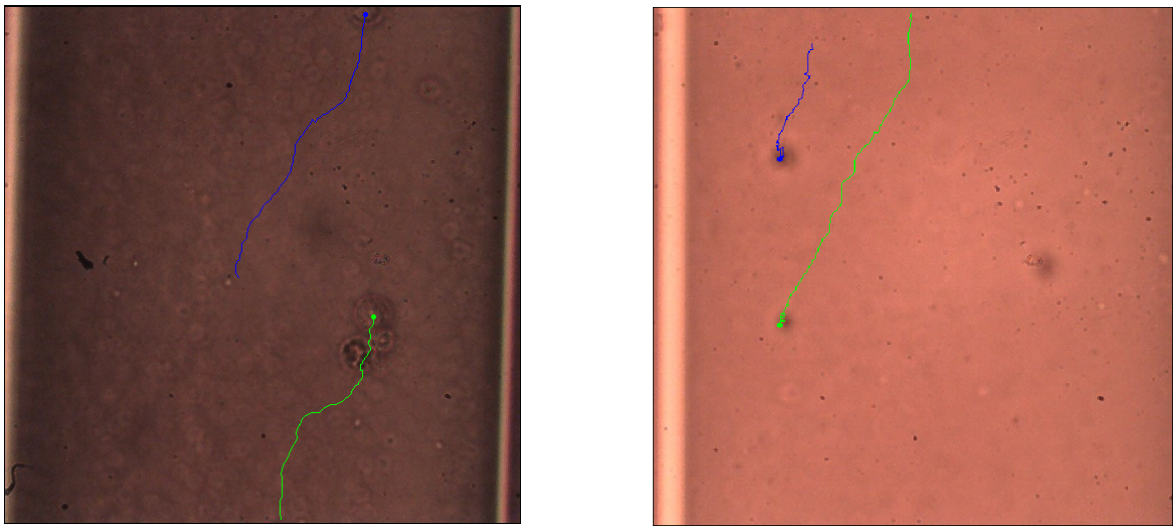


(d)

Figure 4.4: Top view of the movement of red blood cells within the 200 μm inner-diameter capillary after sonication using a 5.681 MHz transducer to the sample. The red blood cells forms a distinct row on left most side of the capillary after being sonicated for a total of 2.73 min. Samples are magnified at 10/20×.

Figure 4.4 shows the slow movement of red blood cells when exposed to ultrasound. After subjecting the sample to sonication over a period of at least two minutes, the cells all gravitate towards certain areas of the capillary. Figure 4.4a is the initial condition of the sample containing just red blood cells prior to any form of sonication applied. This picture will be referenced at 0 s as 2.38 s after the start of this video the transducer is turned on. Figure 4.4b is 20.16 s after the start of sonication and clearly shows more red blood cells coming in focus. This not only indicates that the capillary was in acoustic and visual focus but also that the depth of focus was in the correct layer. This is important otherwise the cells would appear successively blurrier and increasingly difficult to track and identify. After 1.09 min from the start of sonication the image Figure 4.4c shows a clustering of red blood cells to the two edges of the capillary. By Figure 4.4d, 2.73 min after the video started, most of the red blood cells move towards the left side of the capillary and create an almost clear void in the middle-right portion. Outlier red blood cells also convene on the far right side of the capillary indicating the possibility of the same pressure field as the left side or not being able to travel past the pressure point in the middle-right portion.

White blood cells often move in the opposite direction of the red blood cells to reach a different pressure point in the field.



(a) (b)

Figure 4.5: Top view of trajectory of white blood cells under sonication within the capillary of 200 μm inner diameter. Blue and green lines follow the trajectory of the two white blood cells. Dots indicate end points of the trajectories. Samples are magnified at 10/20×.

White blood cell movement, as illustrated in Figure 4.5a, indicates the movement of white blood cells to the right unlike that of the red blood cells, illustrated in Figure 4.4, that moved to the left. Both white blood cells move to the right and in line with each other, confirming the existence of a different pressure area along that specific line parallel to the capillary walls. The confined walls restrict movement and the cells move more freely when there is more dilution of the sample. Higher concentrations of blood produce more white blood cells, however, if the concentration is too high, capillary action and streaming within the cavity are restricted. Figure 4.5b is a special case where the erupted white blood cells form shell-like by-products that initially move in line with the red blood cells but stop when reaching a certain area of the pressure point unlike other red blood cells that carry on moving to the far left sides of the capillary. Despite no longer retaining the qualities of a full intact white blood cell, the cell behaved marginally differently to both white and red blood cells. This indicates the possibility of having multiple pressure channel areas that different types of cells gravitate towards.

Table 4.2: Average final position of the cells after sonication for 2.73 min at different pulse lengths.

Pulse length (μs) per repeat time of 100 ms	Average distance of a cell from the left most capillary wall (μm)		
	Red blood cells	White blood cells	Erupted white blood cells
10.03	94.23	136.91	51.14
35.20	43.24	124.90	56.40

Using quasi-continuous waves enables pulse length variations. The experiment uses pulse length as the portion of the signal on in an interval of 100 ms. The mean final position of red blood cells was far less pronounced at 10.03 μs than at 35.20 μs, averaging at 94.23 μm compared to 43.24 μm respectively as

exhibited in Table 4.2. The average final positions of white blood cells and erupted white blood cells are similar compared to the two pulse lengths. Increasing the pulse length from 35.20 μs resulted in the transducer drawing too much power and failing to work properly thereafter, thus the pulse length was limited to 35.20 μs. Due to the slow reaction time of red blood cells to the 10.03 μs pulse length, 35.20 μs was preferred.

The movement of the white blood cells to the red blood cells differ such that they both travel in opposite directions when sonicated, however, they stream in the capillary if no sonication is present.

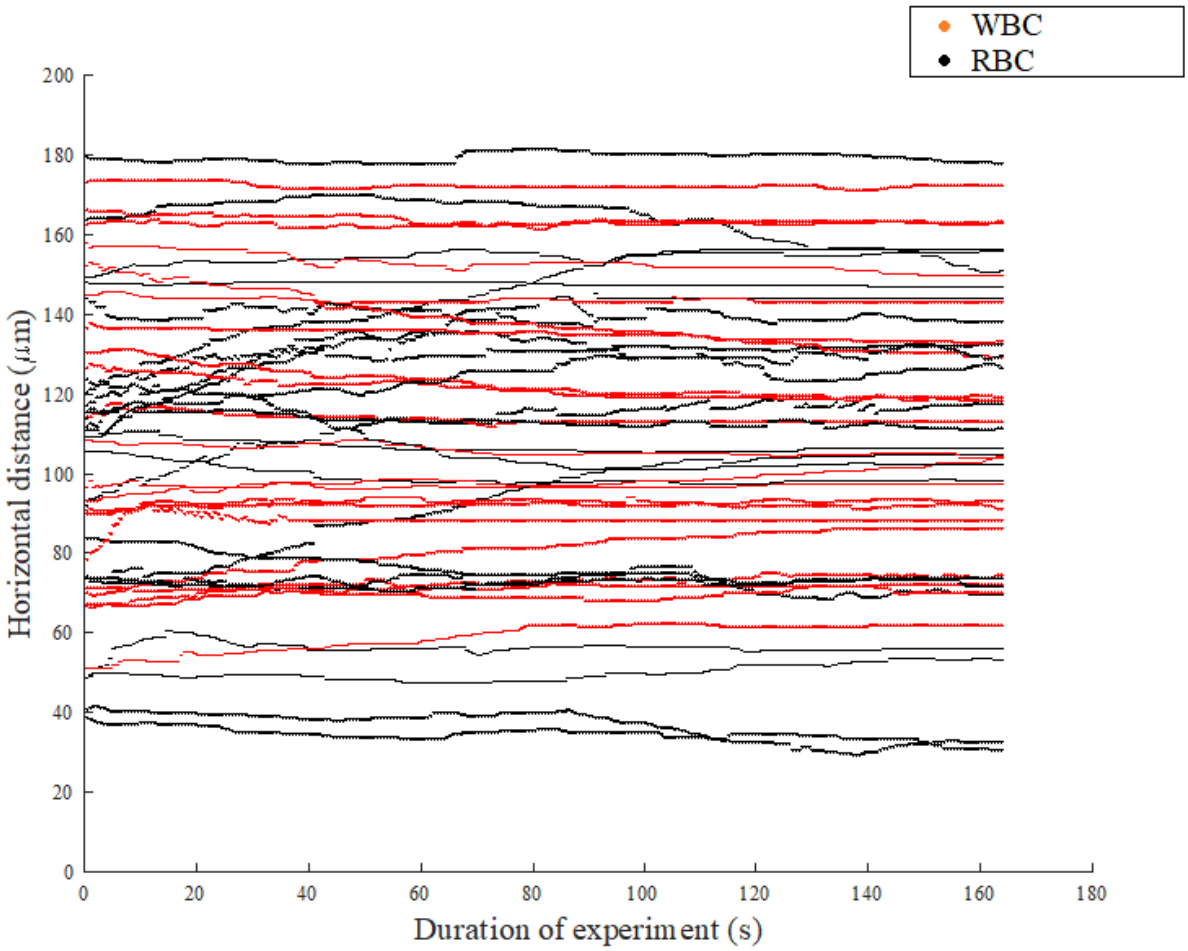


Figure 4.6: Cell trajectory in a field with no applied sonication. 0 μm is the left most side of the capillary wall, chosen as reference. Red dots mark the trajectory of intact and erupted white blood cells and the black dots mark the trajectory of red blood cells.

The cells move vertically with very little horizontal displacement, illustrated in Figure 4.6, whereby the red dots and black dot trajectories are somewhat straight lines regardless of the origin horizontal point. If any movement, it is due to the natural streaming within the capillary.

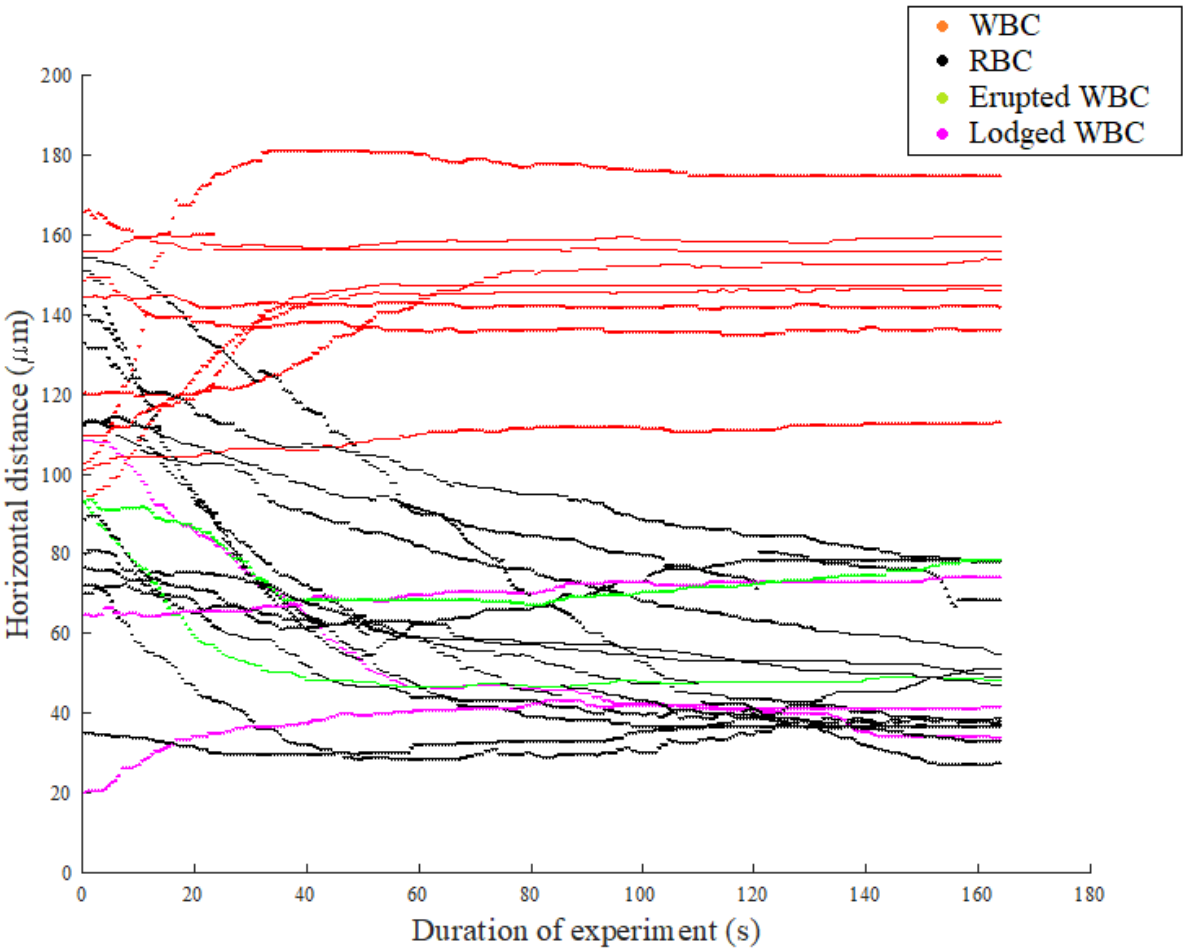


Figure 4.7: Cell trajectory in a field with applied sonication. 0 μm is the left most side of the capillary wall, chosen as reference. Red dots mark the trajectory of white blood cells and the black dots mark the trajectory of red blood cells. Green dots mark early-erupted white blood cells and magenta dots mark white blood cells lodged between moving red blood cells. The sound field on average was turned on within the first 4.2s of the experiment.

Sonication of a sample results in the trajectory of the cells as described in Figure 4.4 and Figure 4.5. White blood cells move to the right side of the capillary and stay in the area when under sonication. This is illustrated in Figure 4.7 which also exhibits how the red blood cells move dramatically from their initial position to the left most side of the capillary. Not indicated in the graph are the residual red blood cells that conglomerate on the far right side of the capillary due to the cells remaining in position and not necessarily moving to the far right side. White blood cells that have initial starting points in the left side of the capillary start moving towards the right before being pushed by the red blood cells which became a strong wall against the white blood cells thus restricting movement. Erupted white blood cells move towards the right before stopping in the 40 μm to 80 μm range.

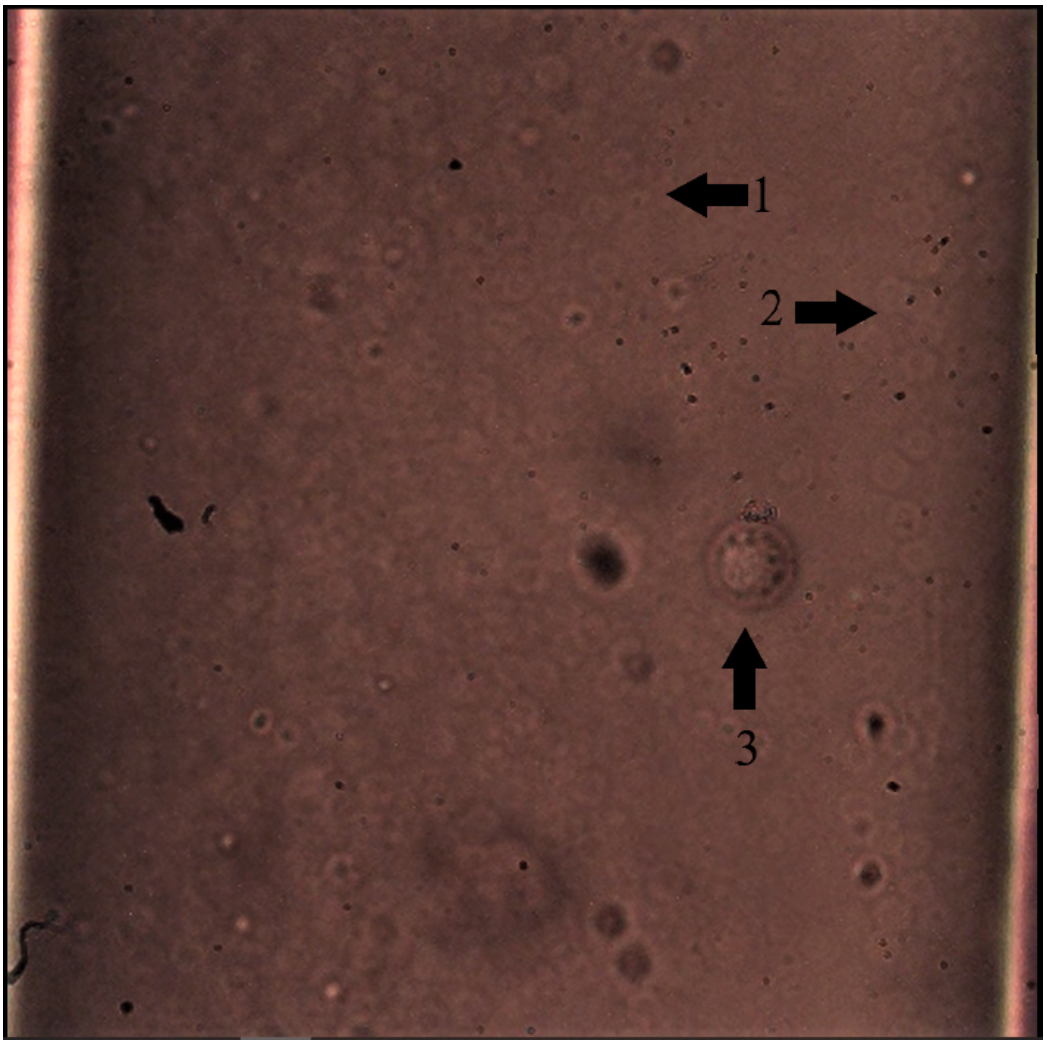


Figure 4.8: Top view of sample composition after sonication for 163.11 s in the 200 μm inner-diameter capillary. Arrow 1 indicates the start of the red blood cells conglomerated in the left side of the capillary. Arrow 2 points to the residual red blood cells on the far right side of the capillary along the wall. Arrow 3 points to the white blood cell on the left side of the capillary. Sample is magnified at 10/20×.

Figure 4.8 indicates the final positions of a sample containing both types of cells, namely red blood cells and white blood cells. Red blood cells generally gravitate towards the left. This is indicated by the arrow labelled 1. There is a clear grouping of red blood cells to the left. The middle-right side contains the white blood cell and the furthermost right side contains the residual red blood cells, indicated by the arrows labelled 3 and 2 respectively.

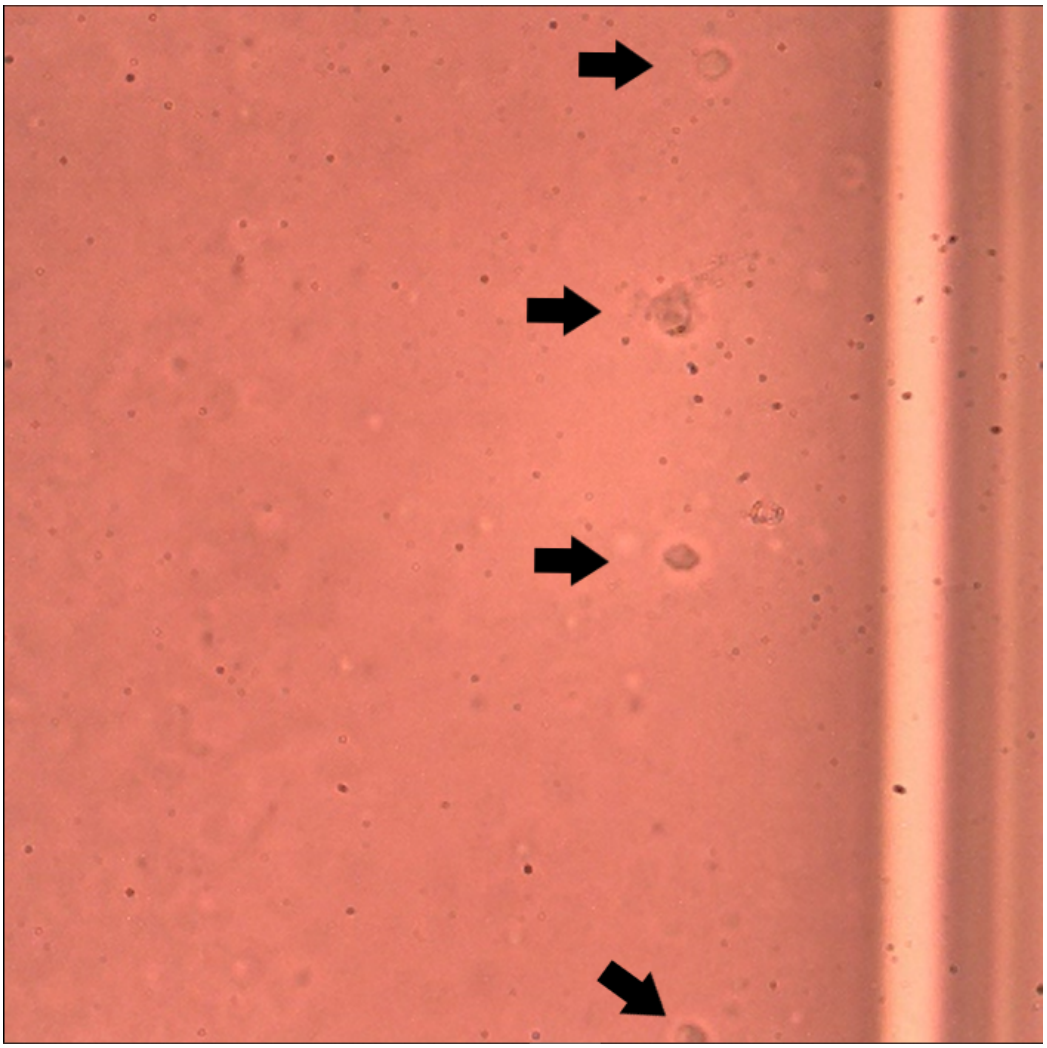


Figure 4.9: Top view of a special case of sonication whereby the white blood cell shells move towards the right of the capillary but stay in a line parallel along the capillary. All four arrows point towards a erupted white blood cells. The sample is moved to show the 200 μm inner-diameter capillary's wall. Sample is magnified at 10/20×.

Figure 4.9 is an instance whereby erupted white blood cells move to the right. The alignment of the cells indicate that they all have similar acoustic properties.

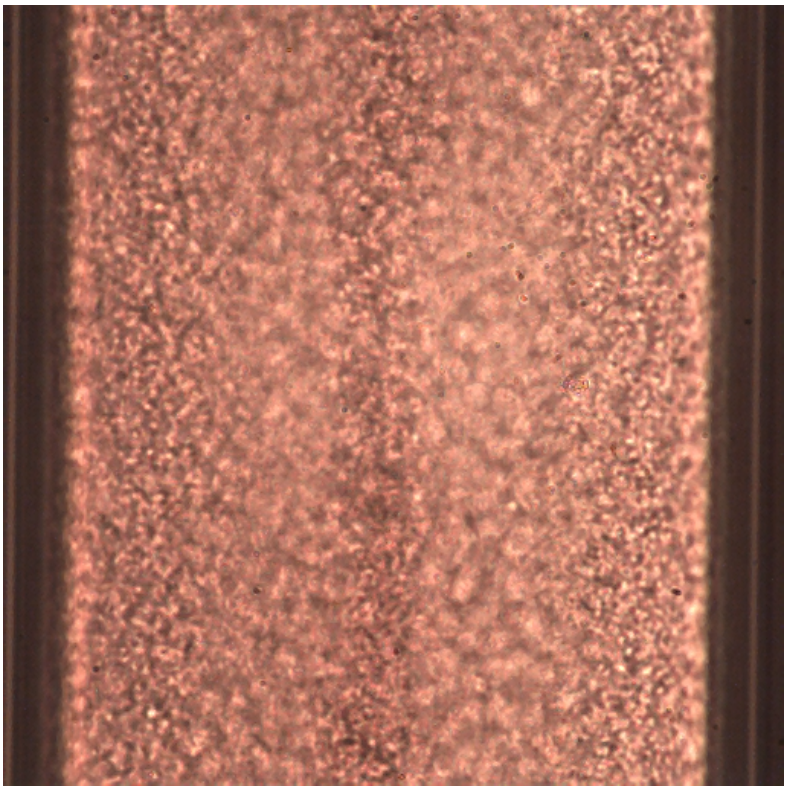


Figure 4.10: Top view of the 200 μm inner-diameter capillary focused at a certain layer indicating a vacuum of cells in two layers and a concentration of cells on the sides and middle of the capillary similar to that of [39]. Sonication for this sample was over eight minutes. Sample is magnified at 10/20×.

Figure 4.10 is an instance in which the capillary was lined up in such a way that sonication produced pressure channels resulting in three channels of red blood cells. This outcome was a mechanism that had red blood cells concentrated in the middle and on the far sides respectively similar to [39] where Figure 4.10 above shows the possibility of such a system recreated in a larger μm model as compared to the smaller microfluidic channel.

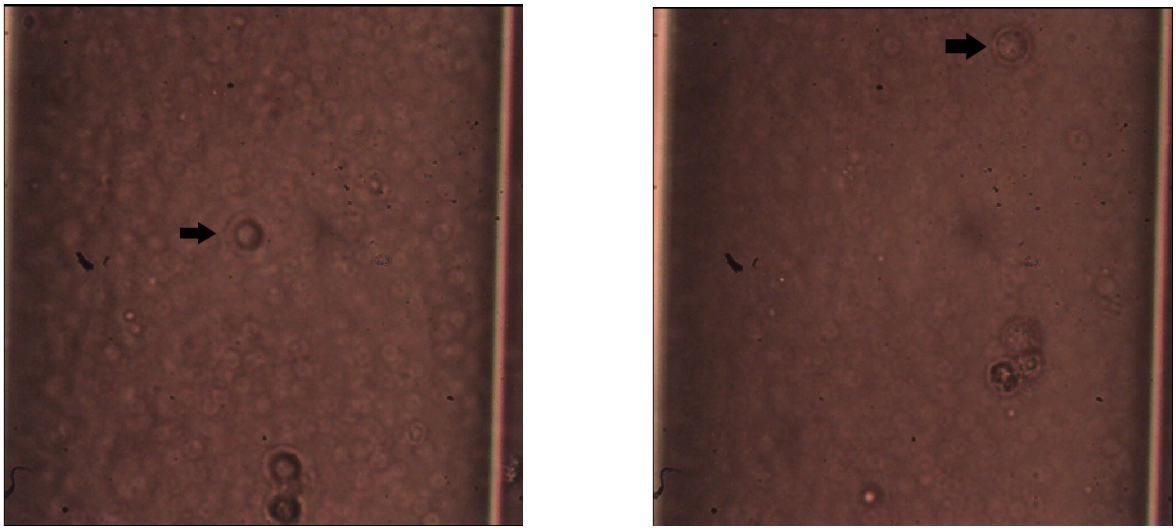
The average final positions of the different cells could be influenced by the acoustic pressure. The range of acoustic pressures in Table 4.3 below correspond to MI value ranges. 0.477 – 0.691 MPa corresponds to 0.2 – 0.29 MI, 0.715 – 0.930 MPa to 0.3 – 0.39 MI and 0.953 – 0.977 MPa to 0.4 – 0.41 MI. The final row is the average final position across all acoustic pressures. There were no erupted white blood cells in the experiments that were in the range of 0.715 – 0.930 MPa. The average position of red blood cells is to the furthest left part of the capillary; the average position of a white blood cell is to the middle-right of the capillary; and the average position of an erupted white blood cell is to the left of the capillary. The average position is due to the capillary not being placed precisely as the previous experiment and having minute variations when being secured across the glass slide. Figure 4.10, is a particular occurrence where the capillary was placed differently than usual resulting in that distinct pattern.

Table 4.3: Average final position of the cells after sonication for 2.73 min at different acoustic pressure ranges.

Acoustic pressure range (MPa)	Average distance of a cell from the left most capillary wall (μm)		
	Red blood cells	White blood cells	Erupted white blood cells
0.477 – 0.691	49.08	140.83	50.31
0.715 – 0.930	30.52	125.78	n/a
0.953 – 0.977	48.93	123.17	63.30
0.477 – 0.977	48.09	125.70	54.64

4.3.2 White blood cell expansion

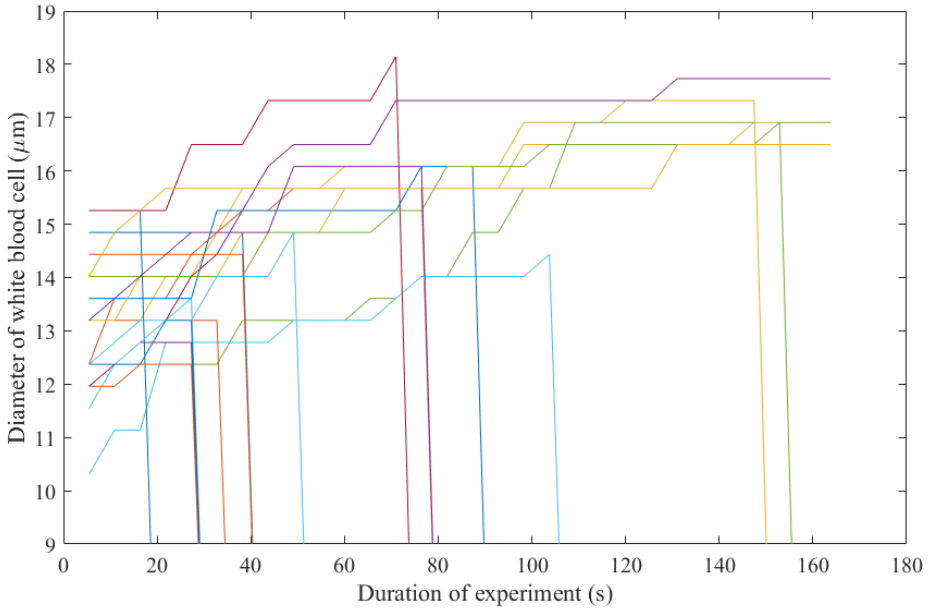
The expansion of white blood cells has been identified as an occurrence in samples which was then observed as being exacerbated with sonication. Membranes of the cells are stretched from initial size before ultimately erupting. The cells enlarge at a faster rate when sonicated than when not.



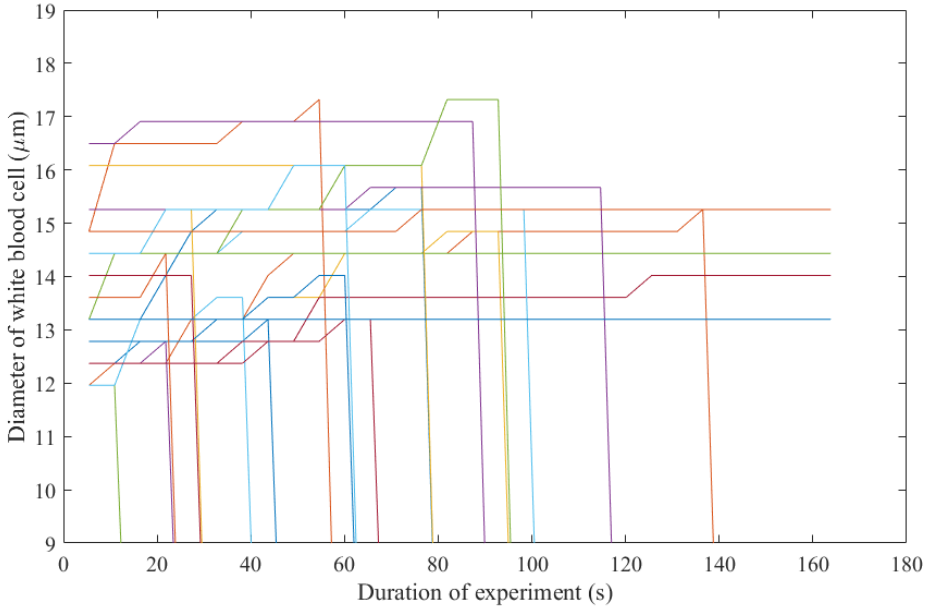
(a) (b)

Figure 4.11: Top view of white blood cell expansion under sonication within the capillary of 200 μm inner diameter. Arrow points to the white blood cell showing clear difference in appearance over 163 s of sonication. Samples are magnified at 10/20×.

White blood cell growth is explicitly visible when the shape, diameter and appearance changes. In Figure 4.11a the cell pointed to was observed to have a thick dark circular inner-line surrounded by a halo-like projection. The cell then moved within the capillary and just before membrane disruption, and ultimately cell eruption, the appearance was slightly different as observed in Figure 4.11b where the thick circular inner-line is thinner and the diameter has increased. There is a visible change in the aesthetics of the cell.



(a)



(b)

Figure 4.12: The growth in size of a white blood cell over 163.11 s. The steep vertical drop occurs when the white blood cell erupts. Each line represents a white blood cell during sonicated experiments (a) and null experiments (b). The different colours represent individual white blood cells to avoid overlapping.

Figure 4.12 compares the change in diameter between sonicated and null experiments. Figure 4.12a represents sonicated experiments that have more upward trajectories of the different multiple curves as compared to Figure 4.12b representing the null experiments. The non-sonicated experiments portray generally gradual upward trajectories with a spread out set of eruption times as expressed by the sudden vertical drop of the line.

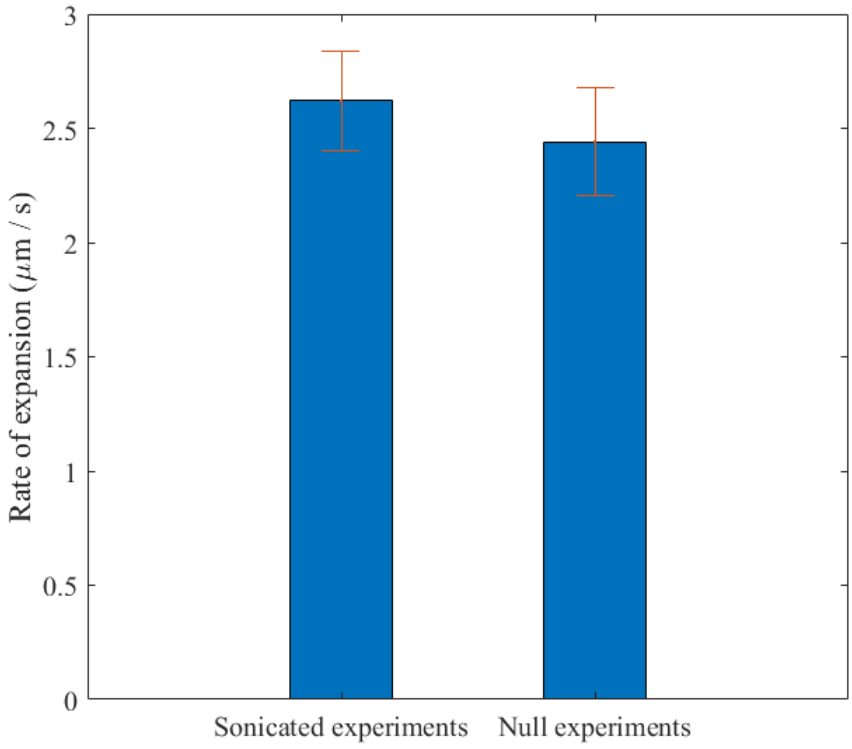


Figure 4.13: A comparison between the average rate of expansion of the diameter between a sonicated and non-sonicated null experiment. The average rate of expansion for a sonicated white blood cell is 2.62 μm/s with an error of 0.2187 μm/s. The average rate of expansion for a null experiment white blood cell is 2.44 μm/s with an error of 0.236 μm/s.

Non-sonicated cells erupt at an average rate of 2.44 μm/s, however, sonicated experiments erupt at an average rate of 2.62 μm/s which is faster than when not sonicated. Non-sonicated experiments have a marginally larger error value indicating a scattered set of expansion rates per experiment.

4.3.3 White blood cell eruption

Another phenomenon observed in the experimentation process was how the white blood cells erupt in the capillary. The effect of ultrasound is observed to exacerbate this effect.

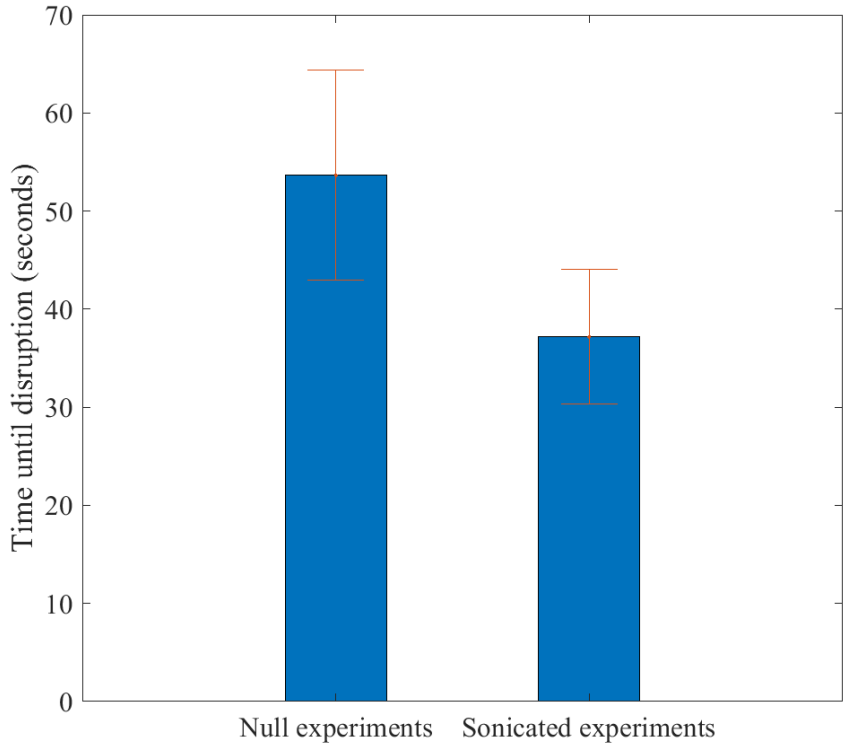
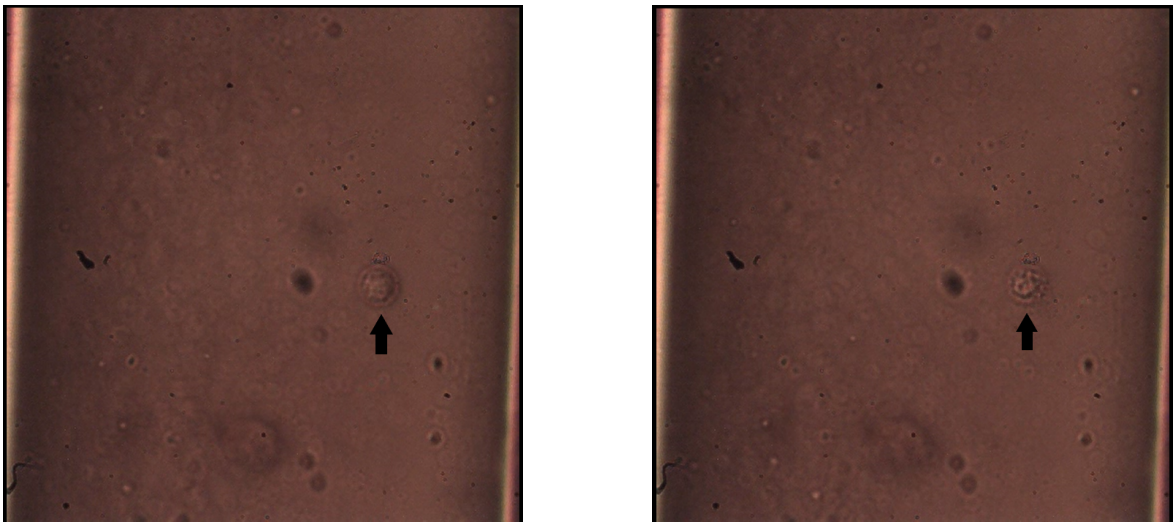


Figure 4.14: A comparison between the average time taken for the eruption of a white blood cell in a sample. The average time for a null experiment is 53.64 s, with an error of 10.71 s, and the average time for a sonicated experiment is 37.17 s, with an error of 6.85 s.

Figure 4.14 compares the average time taken for a white blood cell's eruption in a sample. Samples contained cells in stand still as well as streaming states. The error bar of null experiments are higher, with a standard deviation of 10.71 s, indicating that a larger spread of data for the time taken for eruption. A larger spread of data results in the large uncertainty value. The non-sonicated experiments show various number of durations at which it takes for a white blood cell to erupt. A smaller spread of data, like that of sonicated experiments, indicate a more uniform time frame where most white blood cells took an average of 37.17 s.



(a) (b)

Figure 4.15: Top view comparison of an intact white blood cell and erupted white blood cell within a 200 μm inner-diameter capillary. The black arrow points to the white blood cell before eruption (a) and after eruption (b). Sample is magnified at 10/20×.

An intact white blood cell has a circular shape with a distinct halo-like projection around the cell. The cell is easily identifiable due to the size of the cell being larger than the red blood cells. Another identifying factor of white blood cells is the fact that the cells erupt into a shell, however, sometimes the cells are able to withstand sonication or streaming and not erupt. In such cases the cell size and aura become the distinctive features. Arrow in Figure 4.15a points to the intact cell which has a disrupted membrane in Figure 4.15b, indicating that the cell has erupted. This cell is no longer circular and the contents of the cell are released into the blood stream. The visual presentation of the discharge process can only be clearly observed in video format.

There is a clear distinction between the behaviour of red blood cells, white blood cells and erupted white blood cells in sonicated fields. This includes effects of eruption and expansion of white blood cells which are accelerated in sonicated fields.

Chapter 5

Discussion and Analysis

Summary

This chapter discusses the findings and applications of the project. The results of cell component fractionation using ultrasound are discussed and explained. The experimental results are analysed to provide implementations in the real world that are feasible. The extended application of using the technology as a means to eradicate parasite infested red blood cells are also discussed.

5.1 Result analysis

Ultrasonic blood fractionation, specifically cell separation, was achieved under specific conditions in this study. This effect of ultrasound sonication was expressed in the distinct movements of red and white blood cells, and extended to white blood cell expansion and white blood cell eruption under sonication.

The sample blood used was that from blood banks, thus properties of most of the cells change over time in storage. This is identified from the prevalence of acanthocytes, spiked spherical red blood cells, and an issue posed due to the indication of these different shapes is a possible change in the acoustic properties of blood. Despite these different cells being part of the sample, the red blood cells and cells smaller than the white blood cells tend to gravitate towards the sides of the capillary and behave similarly. The behaviour of these cells are based on the fact that the field of view can only distinguish what are white blood cells and other cells - presumably red blood cells. Even the different types of white blood cells are difficult to distinguish due to the streaming of the sample and lack of adequate focus to identify distinguishing features that are otherwise easily identified in stained smears. Erupted white blood cells have the remaining cell membrane contributing to swift identification. Of the three working transducers, only the 5.681 MHz transducer produced a significant change in the samples when sonicated. The research objective has been restricted in being explored further. The frequency range was restricted to 5.681 MHz. To further separate the cells into their different variations either a larger cavity or more refined transducers need to be used. The effect of the remaining transducers on samples were insignificant and owed to the possibility that they stopped working and possibly resulted in the diminishing level of output for the 285 kHz transducer, as an example. The transducer connection wires were flimsy and this could be a possible contributing factor to the sub-par performance. The connection in conjunction with the deteriorating piezoelectric effects could be possible contributing factors

to the remaining transducers not performing optimally. Different frequencies need to be investigated by producing quality working transducers that can vastly expand the range of frequencies that can be investigated. In this study only the 5.681 MHz transducer resulted in quality results restricting the working field to this frequency.

Pulse length can be manipulated when the number of cycles in an interval is increased. The quasi-continuous frequency of a fixed 100 ms pulse width provided a field of working conditions suitable to implement an induced change in pulse length. Pulse length, in this context, is the percentage of the signal that is on in the interval the signal is applied. A change in pulse length, exhibited in Table 4.2, has a smaller effect in the average final position of a white blood cell and erupted white blood cell than on an average red blood cell. A higher pulse length resulted in the white blood cell marginally travelling closer to the left, from 136.91 μm at 10.03 μs to 124.90 μm at 35.20 μs. The cells are closer to final position of 125.70 μm, as exhibited in Table 4.3, when sonicated at the latter pulse length. This shift indicates that the cells have the possibility of moving more if they were to be exposed for longer durations at a pulse length of 10.03 μs. This hypothesis was not validated in this study and as such needs to be further explored. Erupted white blood cells average at a position of 51.14 μm at a pulse length of 10.03 μs compared to 56.40 μm at a pulse length of 35.20 μs. The irregularity of this finding is based on the small sample space of erupted blood cells that were available to study, however, the cells do not vary too drastically from the average final position of the erupted white blood cell of 54.65 μm, as summarised in Table 4.3. The phenomena of erupted white blood cell particles having its own acoustic properties, however, opens an avenue of finding that more than two types of cells can be separated at a time. The final average position of red blood cells was at 94.23 μm at a pulse length of 10.03 μs compared to 43.24 μm at a pulse length of 35.20 μs. This is a very large discrepancy in final average positions, however, the final position at 35.20 μs corresponds more than the average final position of the red blood cell, in Table 4.3 of 48.09 μm. A feasible reason for this behaviour is based on the physical properties of the cells. Red blood cells are more dense in comparison to white blood cells and erupted white blood cells. The reaction time of the red blood cells can thus be severely affected. Increasing the pulse length forces the red blood cells to move more to the left side of the capillary. Prolonged exposure to a 10.03 μs pulse length needs to be explored to see if the cells move closer to the capillary walls. The high-speed camera can only collect close to two minutes worth of data per experiment, and a pulse length of 35.20 μs presented results that moved red blood cells faster than when sonicated at a pulse length of 10.03 μs. Increasing the pulse length was limited due to the physical restrictions of the transducer thus the various acoustic pressures at this pulse length were investigated.

The general areas of conglomeration across the different samples of blood indicate a distinct method of behaviour by the different cells. It is important to note that the capillaries are not placed exactly the same for every experiment, a note to be taken into account for future extensions based on this work. This is a possible reason why there are a range of areas that the red, white and erupted white blood cells move to rather than one specific narrow line. As the position of the capillary changes, so does the field and channel area. The average final positions of red, white and erupted white blood cells are 48.09 μm, 125.70 μm and 54.64 μm respectively, as exhibited in Table 4.3. This spans over a range of acoustic pressures from 0.477 to 0.977 MPa. The restriction was posed as the corresponding MI value ranges going above are harmful in clinical applications. A fixed pulse width offered a stable premise for the acoustic ranges. Cells only start moving considerably at ranges above 0.477 MPa, however, optimally the red and white blood cell final position with respect to the pressure range of 0.953 – 0.977

MPa corresponds closest to the final overall positions of red and white blood cells cells. The clear difference in the final positions of the different cells indicate the effect of sonication to force cells to behave distinctively. The implication of inherent acoustic properties of cells was consolidated in Figure 4.7 where by red, white and erupted white blood cells have specified ranges in the capillary that they move to when sonicated.

Optimal separation was achieved using a frequency of 5.681 MHz and pulse length of 35.20 μs along pressure ranges of 0.953 – 0.977 MPa. Cell movements are much less pronounced in the lower pulse length ranges and acoustic pressure ranges. Longer exposure at these conditions need to be experimented to see if the ranges can force cells to reach final positions similar to results at optimal conditions. The camera can only record for a two-minute time frame period. This creates limitations of the video due to the high-speed camera thus it necessitated a method to find optimal ranges for separation.

The experimental sample of blood is placed in water at room temperature that is unnatural as compared to body temperature and can thus have adverse effects. The possibility of the colder temperature having an effect needs to be investigated with a sample placed in warmer water imitating the effects of being in the body. The only change is the possibility of the increased metabolic rates that occur due to the existing proteins and chemicals being activated in the rise in temperature. The samples were nearing their expiration date of medical transfusions, as mentioned, and could thus contribute to the mechanical structure and elasticity of the cells being severely affected. This needs to be inspected closely by comparing fresh blood samples. The ultimate goal of this field of study is to apply this as in-vivo projects, therefore, aligning the in-vitro results as close as possible becomes an imperative process.

Two extended findings during sonication were of white blood cell expansion and eruption. White blood cell expansion is indicated by the change in aesthetic appearance. The thick distinguished outer layer of the cell is visibly thinned out. The cells are observed to be more spherical and a halo-like aura that was initially present around the cell is diminished to a lighter coloured outline. The cell, despite moving in the capillary, is easy to track due to the size difference and only behaves differently to an intact white blood cell once it erupts into a shell. The contents of the cell are then discharged once the cell erupts. The solid inner colour of the cell at initial position in Figure 4.11a becomes more granulated in Figure 4.11b. This granulated figure is a stand-still image of bubbling within the cell, however, this effect was not confirmed and needs to be explored in detail for thorough analysis. The distinct patterns in the line graphs, Figure 4.12, describing the expansion of the cells indicate the difference comparing sonicated white blood cells and non-sonicated white blood cells. Figure 4.12a has sharp upward zig-zag patterns for multiple cells indicating steady expansion of cells within the sonicated field. Steep vertical lines indicate that the cells predominantly erupt in the first 100 seconds and are spread out very closely to each other. Eruption time is often overlapping, however, a larger sample space of the time interval can give a different outcome. More in depth analysis needs to be investigated to corroborate this evidence. Samples of freshly drawn blood can retain the mechanical properties of the cells to see if the expansion of the cell is influenced by the presence of a sound field. The error value of 0.2187 μm/s for a sonicated value, as compared to an error value of 0.236 μm/s for a null experiment, indicates the prominent difference in the rate of diameter expansion. The larger uncertainty is caused by the scattered values of the mean rate of expansion for the cell under null experiments. Null experiments have cells standing still or streaming to one end of the capillary depending on the sample, a factor that was uncontrollable. The

effects of streaming and microfluidic effects could be translated to the results as the cells are forced in a 200 μm diameter cavity. The possibility of the influence of the mechanical structure of the cell arises as there are cases of the cells not erupting in either a sonicated or non-sonicated field. The strength and elasticity of the cell layer is responsible for the collapse of the cell. The average rate of expansion for a sonicated cell is 2.62 μm/s, however, this change is for cells that usually expands until ultimately they erupt. The cells erupt faster when sonicated as observed in Figure 4.12a where the vertical drops are concentrated in the first half of the time frame. This is also supported in Figure 4.14, where the mean time of eruption for a sonicated cell is lower than that of a non-sonicated null experiment cell.

White blood cells erupting in a sonicated field indicate the possibility of cell destruction of certain types. The red blood cells, however, did not have significant change unless left in the field for over ten minutes. This is indicated in the Figure 4.10 where by there is a vacuum of cells in two channels but the red blood cells no longer resemble the size, shape and colour observed in Figure 4.4. The cells are clustered and the surrounding liquid seems to have a red tinge indicating red blood cell lysis. More tests using freshly drawn blood need to be used to verify these findings as the current findings are on aged samples of blood. A phenomena observed in the experimentation process is the clear eruption of white blood cells in an acoustic field, thus an indication of the possibility of sonication having an effect on the cells. Sonicated white blood cells erupt on average after 37.17 s and have an uncertainty of 6.85 s. White blood cells during null experiments erupt on average after 53.64 s and have an uncertainty of 10.71 s. The wider error indicates a larger distribution of eruption times of the cells. The difference indicates a clear discrepancy between sonicated and non-sonicated cells. The 16 s average difference indicates the possibility of sonication abetting streaming forces that assist in the white blood cell erupting faster. This is significantly faster than average. The uncertainty of sonicated experiments are lower than that of the null experiments despite being over a range of MI values from 0.2 to around 0.4. The eruption of the white blood cells mirrored the jetting phenomena of microbubbles [67] as the cells discharged the inner contents to the surrounding media. The disrupted portion of the membrane released the cell contents and formed a shell-like residual shape. The samples also had cells previously erupted white blood cells, indicating that the cells membranes of the blood cells are weaker than other cells. The significance of the different time frames of white blood cell eruption can be attributed to the shear forces present on the membrane of the cell. Studies focusing on the effect of different MI values in conjunction with the cells' shear properties with respect to the dynamics of blood-flow can give more insight into the disruption times of white blood cells. Irregular eruption times can also mean that the white blood cells in the bags of blood are unstable and that old cells erupt based solely on the biology of the cell. The bags used from the SANBS treats the donated specimens in such a way that a good portion of the white blood cells are removed, hence the difficulty in finding samples containing white blood cells. The remaining cells could be damaged to such an extent that the behaviour no longer depends on external factors such as sonication, thus movement as simple as streaming in the confined walls of the capillary can cause rupture to the membrane. The quality of the white blood cells may be different per sample and streaming exerts forces on the cell membranes such that the cells have shear forces acting on the membrane. Certain white blood cells do not erupt and this may be attributed to the membrane strength as it is the rupture of this membrane that causes the cell to erupt.

The large uncertainties of both phenomena can be due to the age and storage conditions of the blood being used. Blood banks treat the donor blood further which can have effects on the blood such that the properties can be manipulated to result in undesirable outcomes. The results need to be verified

and refined with whole blood samples that are freshly extracted from donors. Fresh blood can result in new findings that can either corroborate or better explain the phenomenon. More development in the study of the mechanical model of healthy white blood cells can help with the understanding of the different behavioural qualities. Subjecting healthy white blood cells to different ranges of MI values can be a starting point. The importance of healthy cells means that the cells are disturbed as little as possible without external treatments and it behaves as closely to what happens within the body as possible. Studying behaviours of cells in an environment close to the natural state means applications can be catered to in-vivo technology.

Blood provided by the SANBS has a life of twenty-four hours after puncturing the bag until it becomes sedimented, coagulated and useless. The twenty-four hour preliminary study indicated drastic change in behaviour as time of the bag was kept open. Each experiment takes an average of twelve minutes to complete including saving the video. This gave minimal time with the given sample, thus most experiments had different biological factors that could have contributed to the change in behaviour as identified in 4.3.3 that discusses this as well. The cells behave differently due to the sample of blood used. Each sample given to use has the same anticoagulants and preservatives, however, the actual blood within the short bleed bags might have different levels and thus the ratio of the preservatives to the blood vary from bag to bag. Another important factor is the low concentration of white blood cells in the sample. This is indicative of a possible centrifugation or leukocytapheresis (removal of white blood cell) process that has adverse effects on the remaining cells. A positive factor that transcends these limitations is that the translation of the cells in a sonicated field remained similar regardless of the sample. This means that the effect of sonication is constant regardless of the sample of blood used. The donated blood bags also have predetermined amounts of anticoagulants that can adversely affect the natural behaviour of cells. The long time storage of the blood is indicated by the large quantities of acanthocytes. The smears of the contents indicated a presence of platelets and cells which indicates good preservation of cells. Introducing certain chemicals have the possibility of restarting the coagulation process that had been inhibited by the anticoagulants. This may not necessarily mirror the effects of fresh blood as the sample is old, but it could give a similar effect. The cells within the blood bags have been kept below 10°C such that the cell elastic properties may have deteriorated. The mechanical effects of storage need to be verified with fresh samples so that correct applications can be further investigated. Simulations provide a framework where every aspect of an investigation can be micromanaged such that the results reflect a controlled outcome. Preset conditions of the behaviour of the cell can be fed into the simulation such that the characteristics of the feedback can be extrapolated and vice versa. In this study, the biological changes posed various constraints on the outcome due to multiple factors being unknown starting with the identity of the blood sample and the medical history associated with the individual that could have possible outcomes to the study. Each sample of blood was from an anonymous donor, thus the medical history and any other factor associated that could possibly have an effect on the quality of the cells are unknown.

The human capillaries have the slowest flow of blood to allow gaseous exchange within the body. The ample time provided for the process means that this area can be optimum when applying technologies that incorporate in-vivo use of the separation process where particles can be moved and separated within the capillary. The solution of the experiment incorporates a dilution technique to lessen the saturation of cells in the sample. This needs to be addressed in an in-vivo method as there will be no dilution of samples. The microfluidic approach is also not feasible as it relies on cell-by-cell separation

which cannot necessarily be applied due to the large number of capillaries in one area of the body at one time. The experimental method did not incorporate a solid method to control the streaming and consequently the speed of streaming and this might have had an effect on the outcomes. Filling the capillary three-quarters of the way using a marking as a guide can roughly control the speed, however, human error can cause inconsistencies. Future developments will need to take into account the streaming forces and as such implement a method to control the speed of capillary action. The experimental capillary needs to be secured on the tank seeing that the thin material snaps easily which poses a risk when sonicated as it moves and jerks around in the pressure field. The capillary needs to be set in such a way that the securing component clamps the ends softly. LEGO® blocks are harsh and only setting the capillary in the dead centre of the grooves ensures the capillary not snapping. The ridge between the interlocking elevation is wide enough for the capillary to be placed slanted and not in line with the objective. The higher magnifications need the capillary to be placed in such a way that it falls in the field of view otherwise the tank itself needs to be moved as the whole. A smaller ridge and softer clamping system ensures the capillary to be placed in the field of view and secure enough that it does not snap. A loose clamp also means that the capillary is affected by the pressure and will move out of focus. The higher magnification and more secure capillary can provide clearer images for better annotation.

To concurrently observe the same sample under areas of sonication and no sonication can be beneficial as the variables that cannot be changed are no longer an issue for the controlled sample. A set-up to view different areas of the same capillary can aid and overcome such a limitation. This can be achieved by having two areas of focus where one is in the field of view of the transducer's filed and one is further along the capillary far enough so that it is away from the field of the transducer. The field of acoustic pressure is defined and restricted to a certain area of the capillary, and the remaining area is seemingly unaffected by the change. This modification can also show if there is the possibility of extended sonication effects on areas not directly affected by the acoustic region thus paving the way for a new study. The traffic caused by the acoustic pressure has the potential to create a bottle neck effect in a flow and the observation-modification can see if this is the case. Being able to view different layers of the same sample can also see the possible effects of three-dimensional pressure fields. The sonicated waves are three-dimensional, thus the pressure channels have the possibility of reflecting this phenomena. This can only be concurred with a set-up that supports this view. The current study already indicates pressure changes, a future study can corroborate if the fields are multi-dimensional.

Pressure fields from the transducer are restricted such that only a small portion of the capillary is sonicated, and the field of view of the camera is further restricted to a portion of the area of the capillary, not allowing a far range of view to observe the immediate effect that sonicated and non-sonicated areas have. The embedded 10× magnification of the CCD poses this restriction, however, this does not necessarily create a negative impact on this specific study as the main objective is to observe the cells in a field of acoustic changes. The limitation is that null experiments need to be conducted on separate samples. The use of one transducer opened an avenue for possible extension of the work for different frequencies. The conditions of transducers deteriorate over time due to the piezoelectric material not performing as efficiently. The other transducers became ineffective thus restricting the study to the use of only one transducer. The preliminary tests indicated the working conditions of the transducers, however, the actual transducers failed to yield results in the acoustic field as they failed to produce significant movement of any cells in the sample. The resulting only viable transducer was the 5.681

MHz transducer with a diameter of 28.1 mm. A larger number of working transducers mean shifting the topic to the optimal frequency that can produce movement.

Manual testing means that the error bar of analysis can be minimised seeing that the tracking needs to be meticulous despite resulting in a time consuming procedure. Using automated methods such as machine learning or artificial intelligence supported forms of automated analyses needs to be on objects that are easily identifiable. The concept of machine learning heavily relies on good feature extraction. The cells in the sample do not qualify for this as the cells move in and out of focus, changing in colour, density, intensity and size which are generally qualities that are used to identify cells. Feature extraction requires distinctive features which the sample cannot provide especially due to the cell movement. The focus is at one layer and cannot be moved to accommodate one cell, as it will have an effect on the other cells and the calibration methods will need to be changed to accommodate this method of testing. Future work that can focus on cells that move in and out of focus can use such technology to see if a model can be made using the movement of cells in sonicated fields.

For blood transfusions, the white blood cell and other non-red blood cell components cause transfusion complications. Any form of such issues can be averted if an external filtration system can extract these cells further. Centrifugation still has residual white blood cells and other unwanted cells thus a two-factor cleansing process can be applied as a means to clarify blood quantities beyond the normal scope. The currently study is an up-scaled version of normal microfluidic studies, however, mass acoustic separation needs extended research until sonication becomes the main source of blood separation as currently there needs to be more research in this field before it can be applied as a mass form of separation like that of bulk centrifugation.

Multiple biological factors beyond the scope of the analysis have to be factored in. To make sure that the studies are not being influenced by biological indices the need to use a preliminary study that will quantify the effects of external biomarkers on the particles when under an ultrasonic force need to be investigated. The addition of biomarkers like that of chemical biomarkers have the effect of changing the composition of cells. In essence, the effect of external biomarkers and the acoustic effect it has on the cells or particles. There are methods that use intrinsice biomarkers, however, biological components are varied and only have average qualities thus the need for external markers that correctly identify specific cells. This can also be extended to dilution solutions of different types to see if they have specific effects on the cells, for example the effect of Tyrodes Buffer diluted cells against phosphate-buffered saline solution diluted cells in an acoustic field.

The closing, application of component fractionation technology can be extended to identifying malaria cells. The identification of red blood cells containing parasites can have its' own acoustic properties. The concept of this is brought about due to the fact that erupted white blood cells behave entirely different from both white blood cells and red blood cells. This means that visible cells, that are also different in properties, can have a general distinct behaviour associated with it. Furthermore, these parasitic cells can be eradicated by means of redirecting them to the area where it can be removed such as a multi-channel set-up with an exit chamber like that of microfluidic devices. This implementation can be extended by means of a dialysis-type of machine to externally remove the cells and reinsert the healthy cells back into circulation.

Chapter 6

Conclusion

Ultrasonic blood fractionation is a viable method of separating whole blood particles as the different cells react distinctly in an ultrasonic field. Optimally, the cells separate at conditions with a frequency of 5.681 MHz using a pulse length of 35.20 μs along pressure ranges of 0.953 -– 0.977 MPa. The possibility of an extended application in an in-vivo setting or as an external dialysis type machine is near and needs adequate testing to devise the necessary technology.

The specialised experimental set-up created the acoustic conditions such that experiment proved the viability of the use of ultrasound as method to fractionate components when sonicated on whole blood samples. Red blood cells and white blood cells have distinct different physical properties that are confirmed through the behaviour of these cells in a sonicated field. The restricted field of focus only allowed the thorough analysis of these two types of cells and can be a starting point for more in depth analysis. Erupted white blood cells become shell-like figures that retain a portion of the qualities of intact white blood cells. The cells no longer behave like the actual cells but they neither behave like red blood cells, thus having its own distinct properties and behaving accordingly. Prolonged ultrasonic exposure of white blood cells also have an effect such that the cells expand and erupt at a faster rate than when no sonication is applied. It is thus understood that sonication can be used as a method to fractionate whole blood samples. To verify these results, the experiments need to be repeated on controlled fresh samples of blood that are not treated with too many anticoagulants.

In addition, the outcome of the experimental results introduce the possibility of a technology that can use a dialysis-like machine that can separate cells in question without damaging the other cells in the stream. The ultimate application of this technology is to present an avenue of discussion for a non-invasive means to target parasite-infested cells, such as malaria cells, within the blood stream using the distinctive intrinsic properties the cells constitute of to manipulate or eradicate them.

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