DEVELOPMENT OF A MODEL TO ASSESS FLUID COMPOSITION USING ACOUSTIC LEVITATION

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## ABSTRACT

Blood, in all its glory, is at the center stage of life. Its circulation is necessary for the continuation of life. Its regulation is performed by multiple organs in the body. Its transportation of molecules fights diseases and replenishes nutrients. Understanding blood as a media for the body's most critical molecular components has been central to the diagnosis of diseases and viruses throughout the centuries.

The morphology of blood, dictated by the components residing within its domain—erythrocytes, leukocytes, platelets, antigens to fight foreign agents such as viruses, and many more molecules, show the potential to be represented by a patterned frequency set. Here, I present a method using acoustic tweezing, a process which utilizes a custom-built acoustic levitator to trap whole fluid samples in nodes created by ultrasound waves, to induce oscillations. Said oscillations are then analyzed spectrally, where it is found that distinct frequencies are resonated by the sample.

The frequencies and the level of amplification change with sample properties, correlating changes in spectra with changes in morphological structure. Such findings led to additional work in creating models to investigate the impact of controlled changes in fluid composition on the spectra. The hope is that spectral data from oscillation patterns of whole blood and other biological fluids can be used for classification purposes in diagnostics without the excessive drawing of whole blood from patients, without the need for blood fractionation, or the need for complex and expensive laboratory analysis, such as those used in immunoassays.

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# INTRODUCTION

As the ancient Jewish texts say, "the life is in the blood"[1]. Human blood has been understood, since the earliest of civilizations, to possess great mystery and power. It is armed with feeling as if it were a being of its own with nerves and a brain—blood "boils" when we are angry and "chills" when we are afraid, and hovers like a spirit, showing its face in the truest moments of weakness.

The blood of martyrs and the youth was believed to have healing power. While titans such as France's King Louis XI failed to use the blood of the youth to stave off certain death[3], another titan in the field of hematology published "An Anatomical Essay on the Motion of the Heart and Blood in Animals"—William Harvey. Going against the accepted theory that blood was made in the liver from digested food and drink, Harvey discovered that the heart pumped blood to the arteries and that the veins brought the blood back to the heart[5]. This led to transfusion experiments, and the failures of these experiments led to the research of Karl Landsteiner[3], who identified blood types and the interactions between them. This, coupled with Landsteiner's later discovery of the Rhesus factor, was the key to mastering the art of blood transfusions[3].

For blood to qualify for transfusion, it must be tested and screened. Blood tests are one of physicians' most resourced tools to diagnose some of the most common medical conditions of patients in the world today[10,22]. A blood test ordered by a physician can deliver information about whether the red blood cells are delivering normal amounts of oxygen to the tissues, how well the blood will clot, what kind of hormones are circulating in your blood vessels and how much, and so much more. Throughout history, blood tests have adapted to the most consequential pathological findings of the scientific world, (e.g. discovery of the human immunodeficiency virus (HIV), for

which blood transfusion screening began in 1985[1]) and have shaped institutional policy in countries around the world[7,8,9].

Although blood tests can offer significant information about a patient's health, the weaving through the medical maze to acquire the information may require several tests that increase medical costs for patients[14]. Overdrawing of blood, which is done to mitigate the possibilities of errors during laboratory tests (such as difficulties in blood fractionation), have been shown to increase the patient's risk for anemia[13,22]. The number of different tests can lead to mishandling of patient's blood. The desire to simplify blood testing by reducing the amount of blood needed to be drawn and tested is still unfulfilled[5].

The Khismatullin Lab at Tulane University has developed a novel, non-contact method for blood coagulation analysis, referred to as "acoustic tweezing rheometry"[16]. The method uses acoustic levitation to measure the rheological properties of fluids, with volume as little as 6 μL[16]. To levitate an object, the force of gravity pulling the object down must be balanced out by an opposing force. Gravity is balanced by acoustic radiation forces, which reach their peak values at specific points in the standing wave field called nodes[17]. At these nodes, the acoustic radiation forces are just enough to balance gravity, causing the object to levitate. The object will then oscillate when the standing wave field surrounding it is modulated by a driving frequency[27].

When the driving frequency imposed on a levitated fluid drop is close to the natural frequency of the fluid drop, the drop will oscillate at a higher amplitude than at other frequencies. This is called resonance. A hypothesis can be made claiming that the appearance of cells and proteins of different sizes and shapes in whole blood, which change the rheological properties of the whole blood, will also change the whole blood's resonant frequencies. For example, viscosity, a classic rheological property, influences the pattern of oscillation. The more viscous the fluid is, the more it will resist these oscillations, and the viscosity of the blood is influenced by the composition of the blood[15]. Viscosity leads to the shift of resonance frequencies[11]. Additionally,

the abnormality of certain cells and proteins in blood is an indicator for blood-related diseases that affect the viscosity of the blood[19] (e.g., thrombosis, anemia, etc.). Therefore, the extraction of these resonant frequencies from the spectrums of the oscillations of the blood drop may provide a pathway to distinguishing healthy whole blood samples from non-healthy ones.

The purpose of this study is to develop a model to match spectral features, such as resonance frequencies, of the oscillations of biological fluids to biological features embedded in such fluids, such as cells. The success of such a model would be able to use spectral features to detect abnormalities in biological morphology in order to diagnose pathological phenomena.

# BACKGROUND

## I. Chemical Composition of Human Blood

Blood is a mixture of cellular components in a fluid called plasma[1]. The plasma houses the major cellular components of the blood. The major cellular components of the blood are erythrocytes (red cells) and leukocytes (white cells)[1]. The major difference between red and white cells, other than their function, is that white cells are nucleated. The third wheel to the red and white cells are platelets[1], which are proteins recruited to plug damaged sites (wounds) in the blood vessel once the white cells have cleared out the invading bacteria.

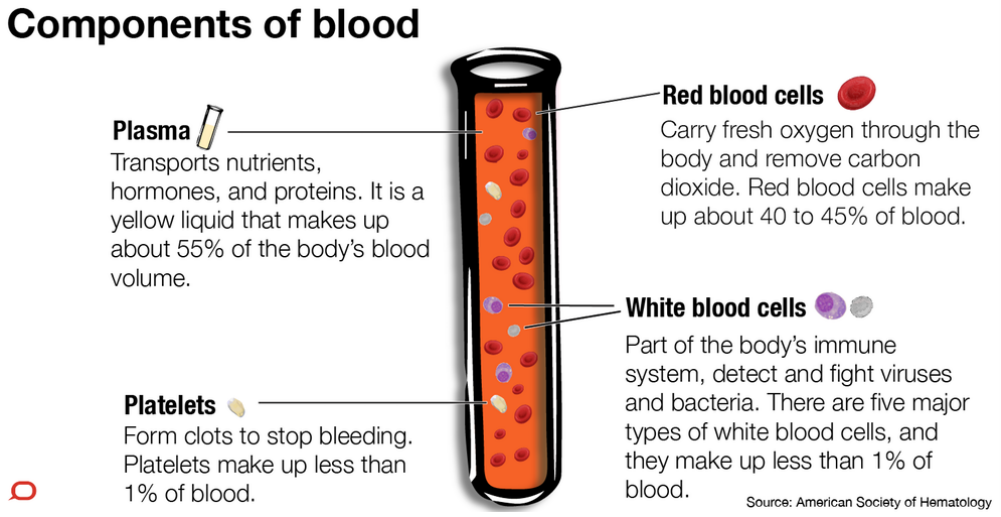


*Figure 1.The three main cellular compositions of whole blood--red blood cells, white blood cells and platelets.*[51]

| Blood Cell | Density[49,58] (g/mL) | Diameter[1,50,57] (μm) |
|---|---|---|
| Red | 1.11 | 7 |
| White | 1.08 | 12-20 |
| Platelets | 1.04-1.08 | 2-3 |

*Table 1. Head-to-head density and size comparisons of red blood cells and white blood cells.*

The function of the red blood cells is to deliver oxygen to tissues in the body. Red cells have a biconcave disk shape[19], which allows them to squeeze into small blood vessels and tight capillary junctions in tissues. A misconfigured red cell shape such as a sickle shape seen in patients with sickle cell anemia[27], where the shape significantly increases the elastic modulus of red cells

and limits the ability to deform, obstructing its circulation[19]. Iron molecules in the proteins called hemoglobin, which have high concentration in red cells, bind oxygen molecules for their delivery into the capillaries.

White cells serve as an important part of the body's immune system[28]. The main three categories of white blood cells are granulocytes (includes basophils, neutrophils, and eosinophils), monocytes and lymphocytes, and their task is to recognize foreign cells in the body and to eliminate them through phagocytosis[1]. During infections, their concentration in the blood increases, as these cells use the blood as a transport means to get to the tissue in need for their aid.

## II. Diagnostic Methods to Assess Fluid Composition

Because blood vessels serve as the transportation system of the body, many different components necessary for life can be extracted from a blood sample and are used for testing. Clinically, the gold standard for assessing the compositions of biological fluids involve some use of immunoassay techniques. Immunoassays work by using immobilized antibodies to capture desired antigens in the testing sample. For higher levels of specificity, another antibody may be used to label the captured antigen, and then a tracer is used to provide the appropriate analytical signal for detection. This technique is used to screen for antibodies or other nanoscale molecules, which can be used to detect viruses or tumor markers in plasma serum (a liquid which separates from blood after it clots).[61] However, immunoassays can generally only be used with specific, expensive and complex instrumentation. Secondly, single-analyte immunoassays do not provide a high enough throughput for clinical usage and are not specific enough to diagnose a specific type of cancer, as a single tumor marker can mark for different types of cancers.[61] The development of multi-analyte immunoassays, which are simple and inexpensive but also keep the specificity and sensitivity of single-analyte immunoassays are of clinical need.

Immunoassays are part of a broader category of a special type of chromatography—affinity chromatography. Affinity chromatography is the use of a biological reaction, such as the bonding of an antibody with an antigen, in order to separate the composition of a liquid.[62] Generally, chromatography is a technique used in organic chemistry and biochemistry applications for separating a substance into its chemical components.[60] A column is packed with a stationary phase element, any element that will react with the desired analyte (sample you are testing). The stationary phase is selected based on the factor (i.e., polarity, size, biochemical binding, boiling point) used for separation. The analyte (mobile phase) will pass through the stationary phase, react, and separate based on the different reactions of its individual components. The simplest example is the use of chromatography to separate the pigment of a leaf or a marker. Once the separation occurs, depending on the factor of separation, may be analyzed for signal detection.[60]

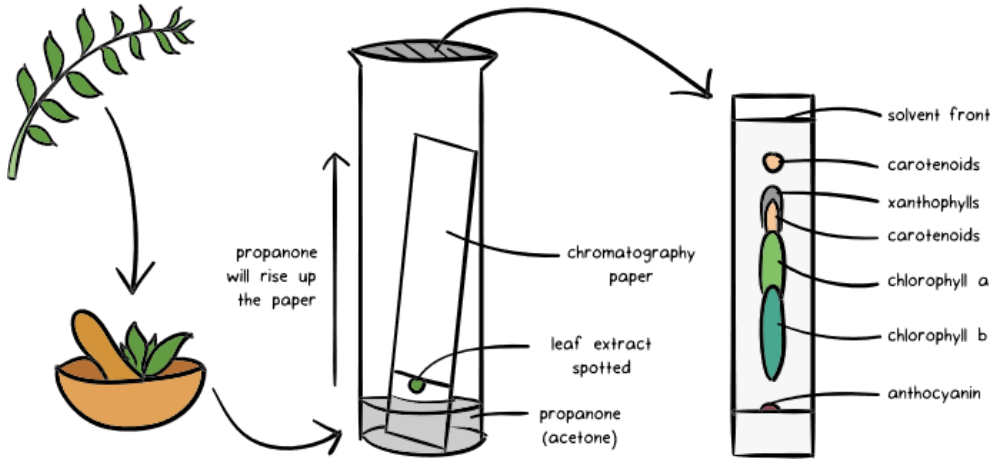


*Figure 2. A simple example of a type of chromatography--paper chromatography. Leaf extract is placed on a strip of paper and will react with propanone. Here, the roles are reserved. In order to reveal the pigmentation of the leaf extract components, the propanone (mobile phase) will rise and move through the extract (stationary phase) and the subsequent reaction reveals the pigment composition.[60]*

Automated technologies to perform analysis of the components of biological fluids widely revolve around flow cytometry coupled with label-detection technologies like spectroscopy.[28] Flow

cytometers can not only be used to diagnose the presence of components on the nanoscale level, but also on the microscale level[34]—blood cells. Hematology analyzers such as Coulter-counters, which applies the principle of particles inducing changes in the impedance when flowing in a tube concurrently with an electrical current, are used to detect the presence of red blood cells and the different types of white blood cells.[66,67] These changes are directly proportional to the particle volume in the assessed fluid.

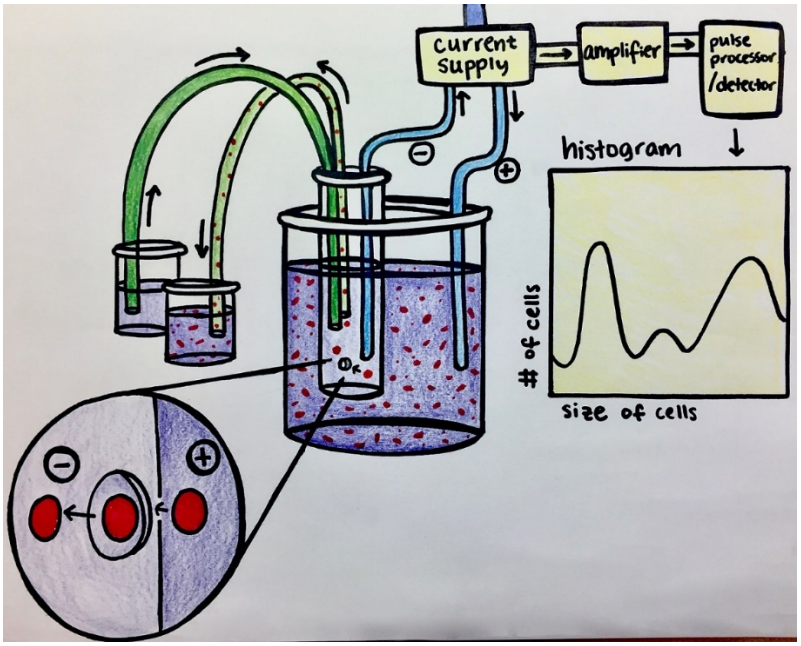


*Figure 3.When a cell passes through electrodes, it will change the acoustic impedance of the system. These changes are then recorded to determine the cell volume, as volume is recorded as a pulse in voltage. This is a reproducible method if the quantity of the suspension is precisely controlled so that values remain the same.[69]*

Because the Coulter-counter doesn't differentiate between living and dead cells or provide an image to validate the cell count or to further enunciate the morphology of the detected cells, flow cytometry light-scattering or fluorescence detection techniques may be coupled with it to supplement its drawbacks to get more detailed information on the cellular components.

Hematology analyzers are beginning to become flow cytometers themselves as analyzers acquire the ability to detect antigen markers.[68] However, the expense of the systems and the

specialization to use the systems create room in the field for future development of apparatuses and methods for upgraded detection and diagnosis.[34]

## III. Diagnostic Methods to Assess Blood Coagulation

When a blood vessel is injured or cut, the body must work quickly to close the tear in the vessel before the hemorrhaging, or bleeding, becomes fatal.[70] The mechanism for this immediate response is called a thrombus, or a blood clot. The blood clot is formed by platelets (also called thrombocytes), which are cells that clump together to form the clot. Through two cascade pathways—extrinsic and intrinsic, a protein called fibrin is formed. Fibrin weaves the mesh of platelet and red blood cells together to plug the wound to prevent further blood loss. The process to activate the formation of the plug, is called coagulation.

Blood clots achieve normal hemostasis, a stop to excessive bleeding. This is achieved when the blood clot is adhered onto the vessel wall at the site of the wound. However, sometimes the blood clot becomes embedded into the lumen of the blood vessel. Clots inside of the lumen can obstruct blood flow in the vessel, like how a serious car accident will stop the flow of traffic. However, in the body, such a stoppage in traffic can be extremely fatal. This is called thrombosis, and in severe cases can lead to ischemia and tissue death.[71] The coagulation cascade pathway's goal is to maintain a fine line between pro- and anti-coagulation to prevent hemorrhaging and thrombosis.[71]

Other examples of coagulation disorders can come from deficiencies in coagulant proteins and their carriers. The factor VIII coagulant protein deficiency is also known as haemophilia A, the factor IX coagulant protein deficiency is known as haemophilia B, and the factor XI coagulant protein deficiency is known as haemophilia C. These deficiencies cause excessive bleeding, and the degree of the deficiency is correlated with the severity of the bleeding.[72] Such factor proteins are monitored through methods described in the next section.

To measure blood coagulation, certain tests are performed to study the interaction of the factors which play a role in the coagulation pathway. Two of these tests are PT and aPTT tests. The Pro-Thrombin (PT) test measures the clotting time of the extrinsic (tissue-factor induced) pathway, while the activated Partial Thromboplastin Time (aPTT) test measures the clotting time of the intrinsic (contact) pathway.[73] Due to a need for laboratories to be able to obtain testing results in time to provide effective anticoagulation treatment, methods to perform these tests have been refined over the years.

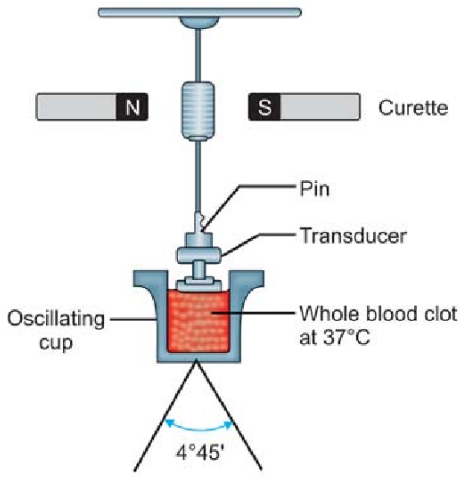


*Figure 4.The thromboelastograph (TEG).*

One of those methods is known as oscillatory rheology. The goal of rheological experiments is to analyze the behavior of fluids under certain applied force. Devices such as the Thromboelastograph (TEG) and the Rotational Thromboelastometer (ROTEM) use this concept of applying an oscillatory shear force to measure the induced shear stress over time. The TEG measures the viscoelasticity of whole blood without the need to separate plasma. The TEG consists of the whole blood being mixed with an activating agent called kaolin and calcium inside of a cup. The cup slowly oscillates around a pin to mimic blood flow, and over a time a clot will form around the pin. During clotting, the torque between the pin and cup is measured. Parameters are taken from the torque curve to classify coagulation status. The TEG has proven useful in applications such as liver surgery, trauma surgery, and obstetrics.[74]

ROTEM, also known as Thromboelastometry (TEM) is like TEG, only that the setup involves the pin oscillating instead of the cup. The ROTEM produces a curve of elasticity over time during clotting. ROTEM is less sensitive to agitation than conventional TEG and provides results in a much quicker time frame.[75]

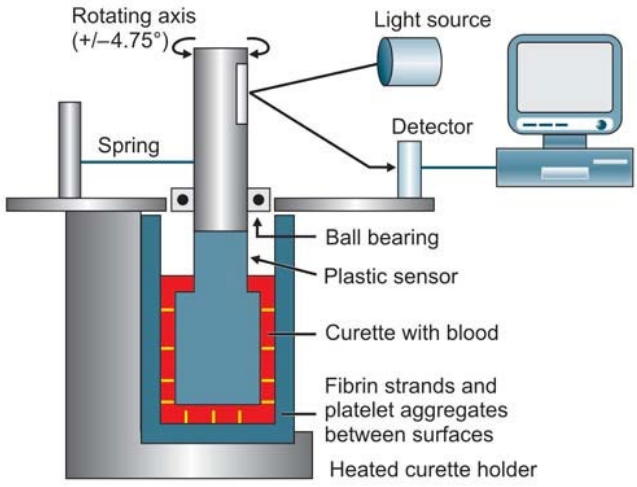


*Figure 5.The rotational thromboelastometer (ROTEM).*

Though the mechanisms described above have been proven useful, there are also some drawbacks to using them. The TEG has been described to have a very large variation coefficient and whole blood specimen samples tend to not have stability. It has been described to have strain amplitude that is uncontrolled and a strain amplitude that exhibits progressive decrease during coagulation. Thus, its operation can delay clot formulation and even modify clot structure during the measurement.[76] Both TEG and ROTEM also require contact between the device used to record the measurements and the fluid sample itself. Such samples may be composed of reactive proteins and polymers and contact with the external environment may lead to reactions that would change the viscoelastic properties of the sample itself.[76]Thus, a non-contact method for the assessment of whole blood samples is needed.

## IV. Acoustic Principles

Sound waves can move through any media. Sound waves are pressure waves, causing particle displacements in the media it travels through. The properties of the media allow it to amplify or dampen the pressure waves. The elasticity of the material determines the ability of the displaced particles to return to their original position—analogous to a guitar string when plucked or a swing when initially pushed[37].

When considering sound waves, however, particle displacement does not occur in an arc-like form like in a swing. Rather, the particles move in the direction of the wave propagation in simple harmonic motion[26]. When displacement in simple harmonic motion occurs, the particle moves at a fixed number of times per second no matter the level of force initially exerted upon it. The displacement and return to original position occur as a cycle of vibration, and the number of cycles per second is referred to as the frequency of vibration. Some objects, such as a tuning fork are designed to vibrate almost exclusively as one frequency[37]. The more complex the object is, the more expansive its frequency set is.

A tuning fork vibrates at one frequency, but most vibrations are more complex than that of a tuning fork[37]. Pressure waves are for the most part, sinusoidal waves, beginning at zero, reaching a maximum peak and minimum peak its cycle before returning to zero. An object like a tuning fork only experiences one pressure wave at a specific frequency. However, the addition of two or more simple sinusoids to one another results in a complex waveform. A complex wave is not a sinusoidal wave, but it is periodic as it repeats itself in a cycle. The lowest frequency at which it repeats is called the fundamental frequency[37].

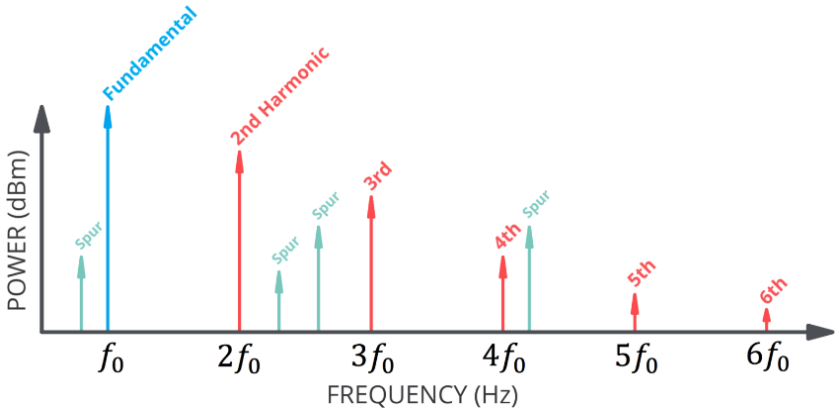


*Figure 6.Multiples of the fundamental frequency are called harmonics. Not every resonant frequency must be a harmonic, however. Spurious peaks may occur that are also very integral to the signal. These are overtones, but not harmonics as they are not multiples of the fundamental.*

The fundamental frequency is also known as the natural resonant frequency, because the fundamental frequency is like a pacemaker—it sets the pace at which other frequencies in a complex tone will follow. All enclosed objects of air, including fluid samples like blood, have natural frequencies. The natural frequency of a system is the frequency at which the system will experience its greatest oscillations when undisturbed by a driving force (when the oscillations are driven by an external force, the frequency of greatest oscillation is called the natural resonant frequency[37]; an example of this would be the frequency of a swing being pushed or the frequency at which a person's voice can break a wine glass). Voice can break a wine glass[26], because when the driving force of the voice's frequency matches the natural resonant frequency of the wine glass, causing maximum vibrations (amplitude), the glass will eventually shatter.

Think of a guitar string which vibrates at a fundamental frequency of 100 Hz. The string's two halves will vibrate at 200 Hz—twice the speed of 100 Hz. The string's thirds will vibrate at three times the speed of the fundamental frequency—300 Hz. These additional frequencies, which occur at multiples of the fundamental, are called harmonics. Together they are referred to as the harmonic series[37]. There is no specific limit to how many vibrating frequencies there are for a specific vibration, but there is a trend that the higher the harmonic, the lower it is in amplitude—therefore, very high harmonics are usually ignored.

In clinical settings, sound waves are used in medicine to produce images of internal structures in the human body. Ultrasound waves (waves with frequencies above the threshold for human hearing—20,000 Hz) are propagated through human tissue by probes placed on the skin called transducers[38]. Perhaps the most popular use of transducers in medical applications is in the determination of the sex of the fetus during pregnancy. Ultrasound-produced images are intensity maps of the sound wave projected back onto the probe by the tissue the wave encounters (some of the wave's intensity is absorbed by the tissue, which will then by absorbed or reflected off by the next tissue the wave encounters)[38]. Biological fluids, including blood have low acoustic impedance and a speed of sound nearly five times that of air, allowing sound waves to easily pass through[39]. The acoustic properties of blood, water, air and polystyrene (a material which will be used to model particles in fluid) are listed in the table below (Table 2).

| Biomaterial | Density ($g/cm^3$)[39,40,59] | Speed of Sound (m/s)[39,40,41,59] | Acoustic Impedance[39,41,59] ($kg/s \cdot m^2$) × $10^6$ |
|---|---|---|---|
| Blood | 1.055 | 1575 | 1.66 |
| Water | 1 | 1480 | 1.5 |
| Air | .0013 | 343 | .0004 |
| Polystyrene | 1.05 | 2450 | 2.52 |

*Table 2.Comparison of acoustic properties of blood, water, air, and polystyrene, a polymer which we will use to mimic molecular components in blood.*

## V. Acoustics in Biomedical Applications

The physical character (i.e. size, shape) of components causing differentiation in acoustic response is a concept heavily used in acoustofluidics. Researchers have designed microfluidic devices capable of separating components from blood samples using strategically placed transducers and flow channel orientation. Ding et. al were able to use the concept of differentiation of acoustic radiation force experienced on particles commonly found in blood samples and designed a microfluidic device capable of separating MCF-7 human breast cancer cells from benign human leukocytes[42]. Wu et. al did the same, combining cell-removal and then exosome-removal modules into one microfluidic invention to separate exosomes from undiluted whole blood samples[43]. The

different experiences in acoustic radiation force will cause, as Ding put it: "different times to migrate to the pressure nodes, thus providing clear identifiers for separation".[42]

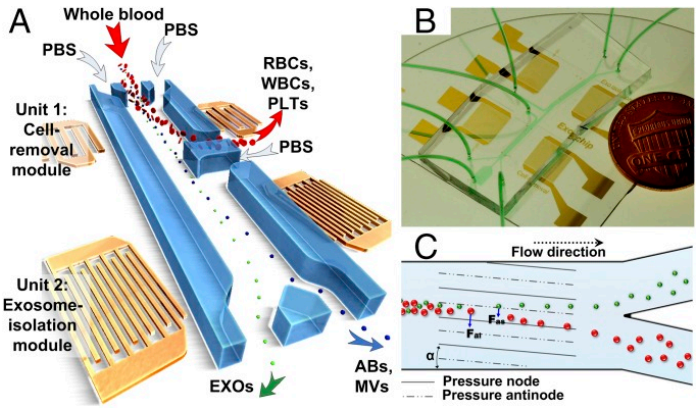


*Figure 7From the Wu paper, (A) schematic of the cell removal and exosome isolation modules, (B) an image of the microfluidic chip itself, (C) mapping of the pressure nodes for the separation of cells.[43] .*

Classification of cellular components by their acoustic properties currently has been performed using photoacoustic methods. Strom et. al developed a microfluidic device embedded with a transducer of 375 MHz center frequency and a pulsed laser of 532 nm focused onto passing cells in the microfluidic flow channels[44]. Their objective was to develop soundwave-based flow cytometer which could assess characteristics of rapidly flowing cells, testing the device on melanoma cells and acute myeloid leukemia cells. The spectral width of the cellular acoustic response to simultaneous ultrasound and photoacoustic wave propagation was used to determine cell diameter of melanoma cells. Strom and Kolios later published another paper using the same ultrasound and photoacoustic techniques (using an acoustic microscope) to differentiate red blood cells, white blood cells, and melanoma cells[45]. In their second study, they found that the white blood cells did not emit a photoacoustic signal, which allowed them to differentiate those cells from melanoma and red blood cells. Because of the uniqueness of cell-types that may be circulating in the bloodstream, the different shapes and sizes may allow for many differentiations of acoustic responses in the same whole blood sample.

## VI. Acoustic Dependence on Viscosity and Elasticity

In studying the effects of acoustic forces on fluid droplets, the droplet's Newtonian (or non-Newtonian) properties must be considered. Whole blood is a non-Newtonian fluid. Unlike Newtonian fluids like water, the viscosity of blood is dependent on the shear rate the blood experiences and is not constant[78]. The biggest contributor to the viscosity of blood are the red blood cells, which aggregate together at low shear rates and deform and move in the direction of flow at high shear rates. It is the deformability of these cells, however, which would make the description of blood as a non-Newtonian, viscous fluid as inaccurate[78]. Blood, rather is a viscoelastic fluid, displaying both properties of viscosity and elasticity.

Aqueous dextran solutions are used as a substitute for blood in this study. Unlike blood, aqueous dextran is not a viscoelastic fluid. Aqueous dextran is instead a Newtonian fluid with a predictable viscosity[77]. With increasing dextran concentration, the viscosity of the droplet increases[77]. Additionally, the attenuation coefficient and acoustic impedance (resistance) of the droplet increases—both are key factors in understanding how ultrasound waves interact with the droplet. Holt et. al found that increased dextran concentrations do not show significant changes in the bulk elasticity of the solution[16]. The acoustic methods used in this study showed insensitivity to changes in the bulk viscosity of the solutions but are sensitive to changes in bulk elasticity of solutions (they levitated other gels and blood samples), indicating that shifts in acoustic properties in the solutions during solidification or coagulation are due to changes in bulk elasticity and not viscosity[16].

Khismatullin and Nadim concluded that under high Reynolds number conditions (low-viscosity condition), elasticity has a minor effect on the shape oscillations of the levitated droplet[79]. The droplet would undergo such oscillations due to surface tension. Lowering the Reynolds number (increasing viscosity) would reduce the oscillation modes in a viscous solution, but in viscoelastic solution, new oscillation modes would arise—these would be caused by viscous and elastic stresses.[79] Research has been, and is still is, being done to further understand the effects of

viscoelasticity on shape oscillations of blood droplets under acoustic forces in the Khismatullin laboratory.

## VII. Acoustic Levitation

By using ultrasound waves to interact with liquids, all the above can be accomplished. Ultrasonic acoustic levitation is achieved by using acoustic tweezers to hold objects in air. Levitation is achieved when the force of gravity pulling the object down is balanced by an opposing force[48]. In this case, the opposing force is an acoustic radiation force. When the tweezers generate a standing wave field, this force reaches its peak values at specific points called nodes in the standing wave field where the amplitude is zero (waves cancel each other out at the nodes when they interact)[47]. At the nodes, the acoustic radiation force is enough to balance gravity, allowing for levitation of the object to occur. These nodes are like the pressure nodes described in the microfluidic applications of acoustic tweezing in the Wu and Ding papers.

Acoustic tweezing has been used in biomedical applications to manipulate cells and biological liquids and is preferred due to its ability to eliminate the potential effects of sample contact with device walls—including contamination or altered measurements[16]. It is also relatively inexpensive compared to the high-powered lasers needed for optical tweezing methods and can be used for interacting with nanometer-sized samples, unlike optical or magnetic tweezers. Unlike other methods, acoustic tweezing has been shown not to damage cellular structure, proving its biocompatibility[17].

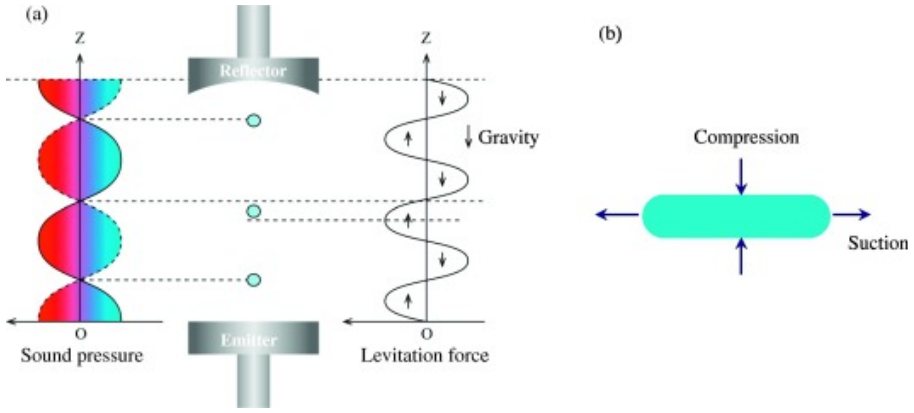


*Figure 8. Effects of acoustic radiation force on levitated liquids.*[55]

The Khismatullin Lab has used acoustic tweezing to study the rheological properties of fluid samples, including whole blood. The lab, in collaboration with researchers from Boston University, has developed a novel, non-contact method for rheological analysis, referred to as "acoustic tweezing rheometry"[16]. The method uses acoustic levitation to measure with rheological properties of fluids, with volume as little as 6 μL with the purpose of developing more accurate blood coagulation tests[16].

Levitation at pressure nodes isolates the fluid sample to be manipulated by a driving force without contamination from the device. In dynamic acoustic tweezing experiments, a modulation signal is introduced to the standing wave field. This modulation signal is analogous to the driving force of air passing through the vocal folds into the vocal tract. The modulation signal induces fluid drop oscillations, which are analogous to the particle displacement (amplitude) due to the pressure wave[27]. The frequencies at which greatest oscillations occur are the resonance frequencies of the system.

Such responses to the modulated signal have been used to differentiate between malaria-afflicted or sickled red blood cells, which are much stiffer than normal red blood cells[19]. Stiffness affects the ability of red blood cells to deform which is, as mentioned previously, an integral part of the cell's ability to squeeze through tissue capillaries. The viscosity of a whole blood sample

also affects the ability of the sample to oscillate. Because whole blood is a non-Newtonian fluid, its resistance to flow is dependent on a non-constant shear rate generated by the interaction between its cellular components[15]. Therefore, the character of these components (mainly red blood cells) directly impacts the oscillation of the whole blood sample.

## VIII. Spectral Analysis

After the signal is recorded, the oscillations of the media due to the presence of a driving force can be analyzed on a spectrum, a graph showing the amplitude response at each frequency in the system. The spectrum is the result of the computation of the Fourier transform of the time-domain signal (time vs. amplitude)[37]. The Fourier transform computes the energy at each frequency. The peaks of the Fourier transform can be identified as the resonant frequencies of the system. Spectrums are useful for a more quantitative look at the relationship between frequency and amplitude, as exact values are easier to extract from spectrums than its counterpart, the spectrogram[37].

As perhaps the most widely used analytical tool in speech science, spectrograms provide information not only about the frequency components of the signal, but also about the corresponding amplitude at those frequencies[26]. This is done by mapping the points on the spectrogram by intensity—normally the more intense portions represent higher amplitudes (this is done using a color map, black and white is most typical). Formants are found on spectrograms by finding broad bands of higher energy. The center of the energy band is chosen as the representative frequency of the formant[37].

Another metric which can be used to measure the quality of the formants beyond their amplitudes, is the quality factor, or Q-Factor[64]. The Q-Factor is found by dividing the frequency of the peak by the width of the peak (the frequencies which corresponding to the beginning and end of the hump the peak creates).[64]

$$Q = \frac{f_0}{BW}$$

*Equation 1. Q-factor is found by dividing the resonant frequency (f0) by the bandwidth of the resonance.*

Q-Factor is a measure of how damped the oscillator is. Damping is the opposite of amplification—instead of reinforcing a signal, dampening lowers the signal quality. The lower the Q-factor, the higher the damping, and the weaker the resonance. In this application, the higher the amplitude and the more prominent the formant is, the higher the Q-factor.[63]

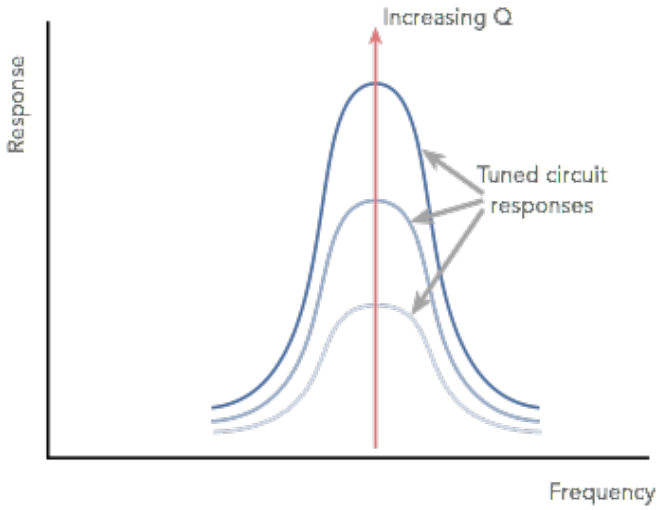


*Figure 9. For a resonant circuit, the higher the Q-factor, the lower the damping and the sharper the resonant peak is.[63]*

The value of the Q-factor describes how damped the system is. A system with a Q-factor > ½ is an has an underdamped response, meaning that the damping is low so the oscillation will be sustained for a longer period, like in a pendulum. A system with a Q-factor < ½ is an overdamped system, and the oscillation of this system is not going to be sustained as long. In fact, the oscillation will exponentially decay. A system with a Q-factor = ½ is a critically damped system, and this system gradually rises to a steady-state. A strong resonance system will have peaks with higher Q-factors[63].

Acoustic levitation coupled with dynamic modulation will be used to induce oscillations of the levitated fluid. Such oscillations will be recorded and then spectrally measured for its spectral components to chart a spectral fingerprint of the fluid.

# MATERIALS AND METHODS

## I. Acoustic Tweezing

To test the hypothesis, a custom-made acoustic levitation system, developed by Levisonics, Inc. (patent owned by Tulane University) was used for the experiments. The core of the device is a transducer and reflector, positioned as far apart from each other as a half-wavelength. The transducer receives the ultrasound signal (this will be referred to as the carrier wave from this point on) from a function generator (Agilent 33220A Series Function Generator, Santa Clara, CA). Prior to receiving the carrier wave, the carrier wave is first amplified after leaving the function generator by an amplifier (Krohn-Hite, Brockton, MA). This signal, once reaching the transducer will create a standing wave field. This standing wave field is made steady by two, 3.175 mm thick piezoelectric discs (Channel Industries, Santa Barbara, CA) serving as a 30 kHz transducer, and an aluminum cylinder serving as a reflector.

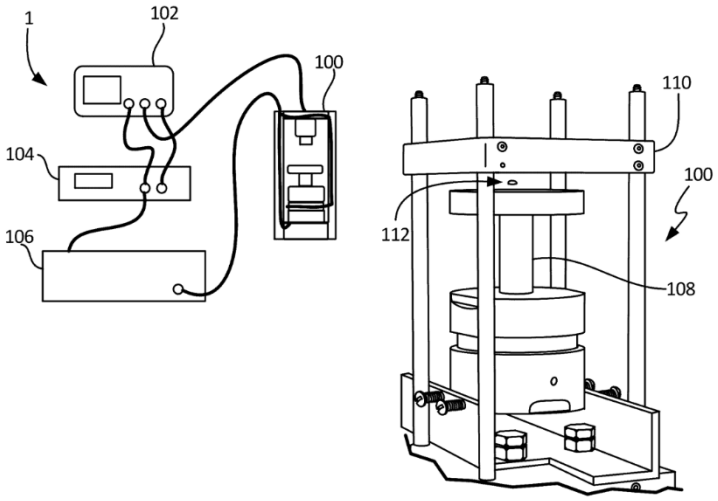


*Figure 10. Schematic of the device developed by the Khismatullin Lab. The device consists of a levitator (100), oscilloscope (102), function generator (104), amplifier (106), transducer (108), reflector (110), and a sample being levitated (112). The carrier wave is sent by the function generator, amplified by the amplifier, and then sent to the transducer.*[56]

In dynamic acoustic tweezing spectroscopy experiments, the amplitude of the carrier wave is modulated by a sine wave using a second function generator. This modulation signal induces oscillations in the fluid drop, which is placed at a pressure node in the standing wave field and prior to the modulation, held steady. The oscillations of the fluid drop are recorded by a photodetector, which is aligned with the fluid drop. A flashlight is used as a light source, aligned with the levitated drop and the photodetector. The flashlight shines light through to the photodetector. As the fluid drop oscillates, it changes the amount of light which reaches the photodetector. Therefore, the output voltage from the photodetector is linearly proportional to the changing area of the fluid drop while oscillating.

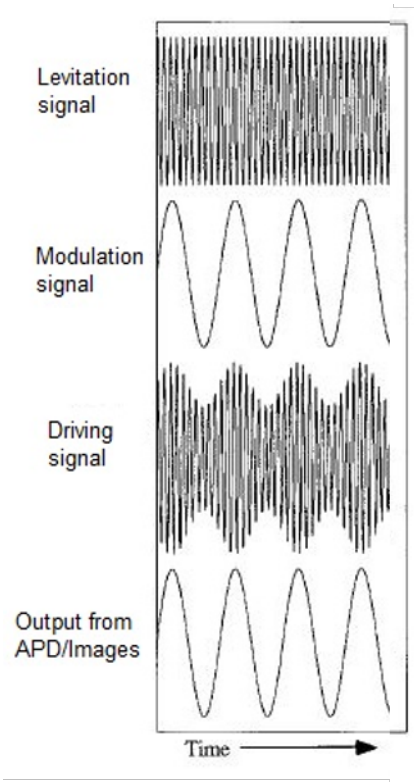


*Figure 11. The driving (carrier) signal is modulated by a sine wave, which serves as the modulation signal. The modulated output induces oscillations from the levitated fluid drop, which are recorded by a photodetector apparatus.*

## II. Sample Preparation

Samples for establishing a proof of concept were prepared by first creating base solutions of 5% dextran (United States Biological, Salem, MA) in aqueous solution. A 5% dextran concentration was chosen due to its ability to induce a most stable oscillatory mode. Dextran, in fact, has been used to make a fluid designed to mimic blood for ultrasonic applications because of dextran's ability to raise the viscosity of the water-based fluid[65]. An increase in polymer concentration, such as dextran, increases the bulk viscosity of the solution.[16] Therefore, fewer modes of oscillation in the solution are induced due to a higher viscosity (which reduces the tendency of the sample to oscillate when induced). Furthermore, increasing dextran concentration does not increase the elasticity of the solution.[16] The ability to reduce the number of variable changes in the system allows for a more stable control group.

Each base solution of 5% dextran was mixed with a 10% solution of polystyrene microparticles (Sigma Aldrich, St. Louis, MO) in order to create solutions with concentrations of .025 M, .05 M, and .1 M polystyrene microparticles in 5% dextran. Droplets from the solutions of these three solutions, along with that of the control group (5% dextran without polystyrene microparticles) were levitated.

## III. Experimental Procedure

The levitation apparatus has two function generators. The first function generator is used to create the standing wave acoustic field, and thus the frequency of the carrier wave must be controlled by the user in order to create a strong enough acoustic field to activate the node necessary to levitate the droplet. Once the node is activated, a pipette was used to extract 6 μL of solution and placed on the node in the standing wave field in-between the transducer and reflector. Once the acoustic field is strong enough to trap the droplet and levitate it, a custom LabVIEW code automatically triggers the second function generator to modulate the acoustic field. The sine wave serving as a modulation signal, modulates the acoustic field at a frequency sweep from 150 Hz to 50 Hz for ten seconds. During this modulation, voltage from the photodetector is captured using a

data acquisition system (put data acquisition system information here). The number of samples levitated are in the table below. The levitation period was done in one-minute intervals for ten minutes, meaning that the modulation signal was sent to the transducer every minute in order to induce ten-second oscillations.

| **Group** | **Samples Levitated** | **Microparticle Concentration** |
|---|---|---|
| 5% Dextran (control) | 7 | None |
| Control w/ .025M PSMP | 7 | Lowest |
| Control w/ .05M PSMP | 7 | Intermediate |
| Control w/ .1M PSMP | 6 | Highest |

*Table 3. 'The number of sample fluid droplets levitated from the control and experimental groups.*

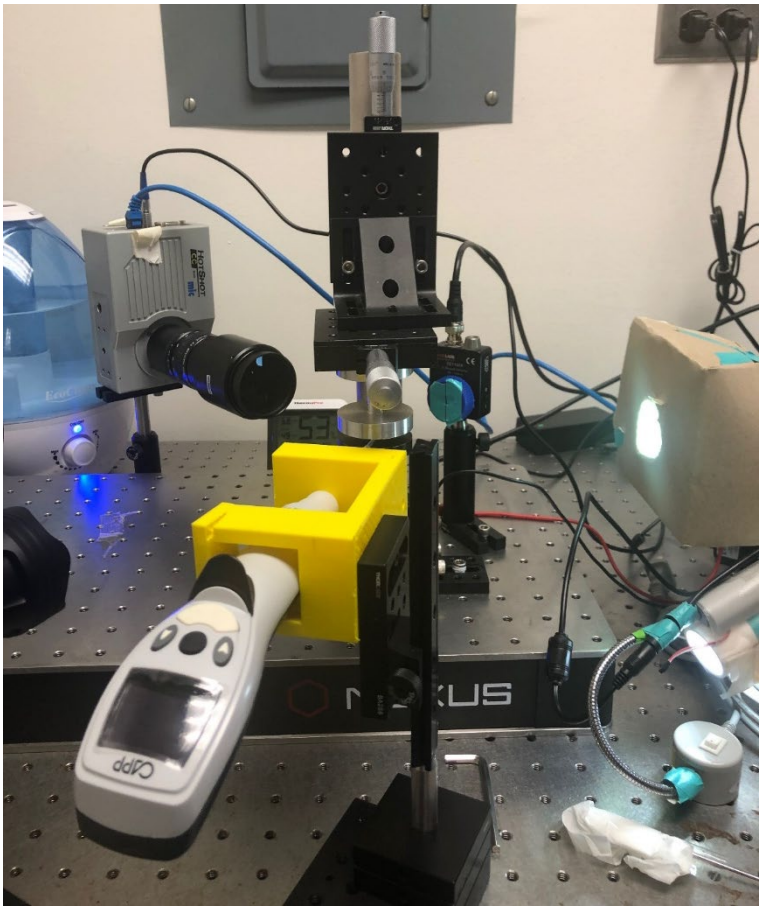

*Figure 12. The levitation system--an automated syringe, placed on a stand is used to inject a 6 μL sample from the solution into the node sandwiched in-between the plates of the levitator—a transducer and a reflector. A flashlight is used as a light source for the camera, which is used to take photographs of the levitated sample. A humidifier (behind the camera) can be used to control the humidity in the room.*

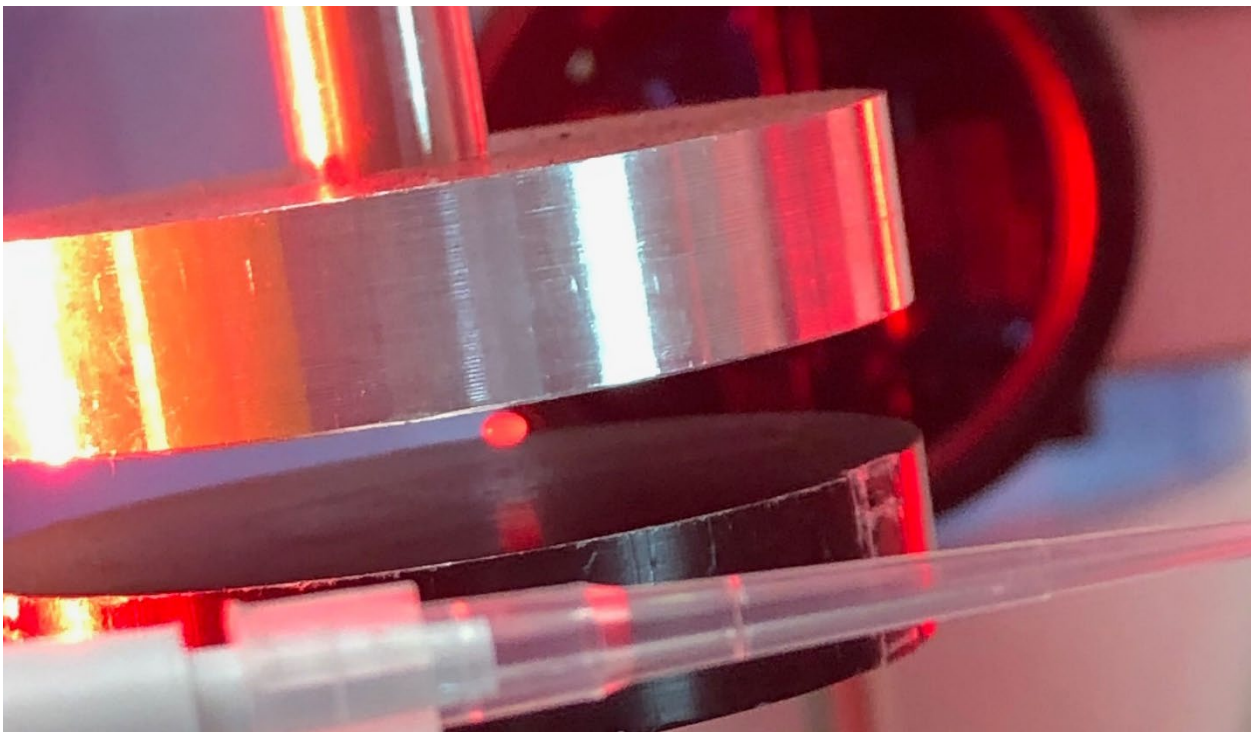

*Figure 13. A fluid sample as small as 6 µL can be levitated using this system. The sample is placed in the path of light from a flashlight. This light shines in a straight line to a photodiode, and the oscillations of the sample will block the light going to the photodiode. The greater the oscillations, the more light is blocked.*

## IV. Signal Processing and Analysis

To analyze the oscillation data of the fluid droplets, a custom MATLAB code was designed with the following goals—first, to successfully filter the noise from the signal (with the most important obstacle being to distinguish noise peaks from actual resonance peaks), secondly, to convert the signal from time-domain to frequency-domain, and thirdly, to extract spectral features from the signal in frequency-domain. The latter was done using a Fast Fourier transform (as referenced in the thesis background). The primary spectral feature extracted for this study was the quality factor of the spectral resonances.

Because of the nature of the experimental set-up, the bandpass filter used for filtering was a narrow band filter—only allowing signal at frequencies between 50 and 150 Hz through the filter and attenuating frequencies outside of this range. In this way, the process for processing and

analyzing data files would be time efficient. Occasionally, small peaks would be seen at the harmonics of 60 Hz, but those peaks were dismissed as the cause of them was electrical interference. As will be explained further in the discussion section of the thesis, problems could arise with resonance frequency bands overlapping with noise peaks. As will be explained further in the discussion section, it was discovered the quality factor also proved to be a useful tool in distinguishing noise peaks from true resonances. The basic code used to achieve these goals will be found in the appendix portion of this thesis.

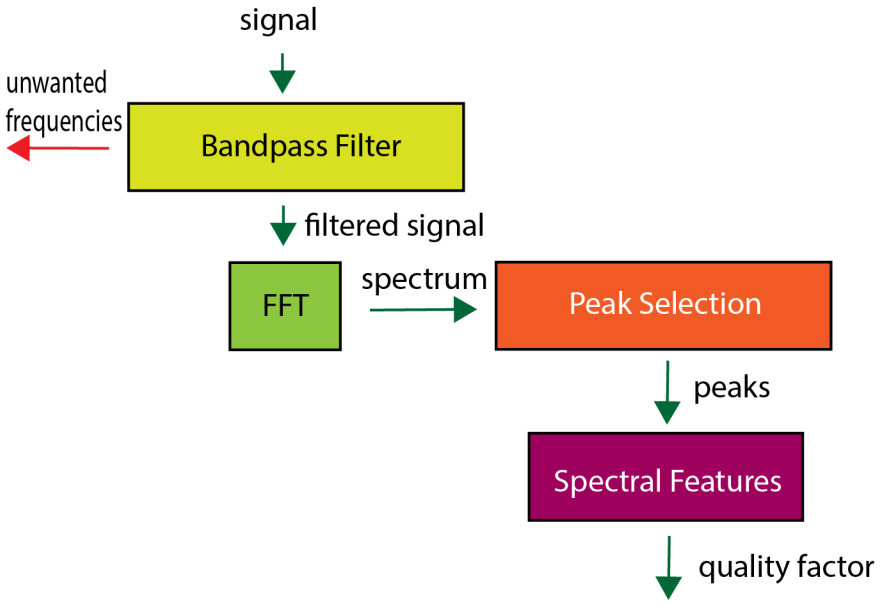


*Figure 14. A block diagram showing the workflow from signal to quality factor. The signal is filtered through a bandpass filter before being transformed to the frequency-domain. Then, resonant peaks are selected and then spectral features are extracted from the peak.*

# RESULTS

## I. Fourier Analysis

The custom MATLAB code received signals akin to the one shown in Figure 1 and successfully used the bandpass filter and Fast Fourier Transform analysis (Figure 2) to extract resonance data from the spectra. Fourier analysis of the drop oscillation response to the frequency modulation in the listed experiments revealed at least one resonance in the oscillation signal for each trial performed in the control and the experimental groups.

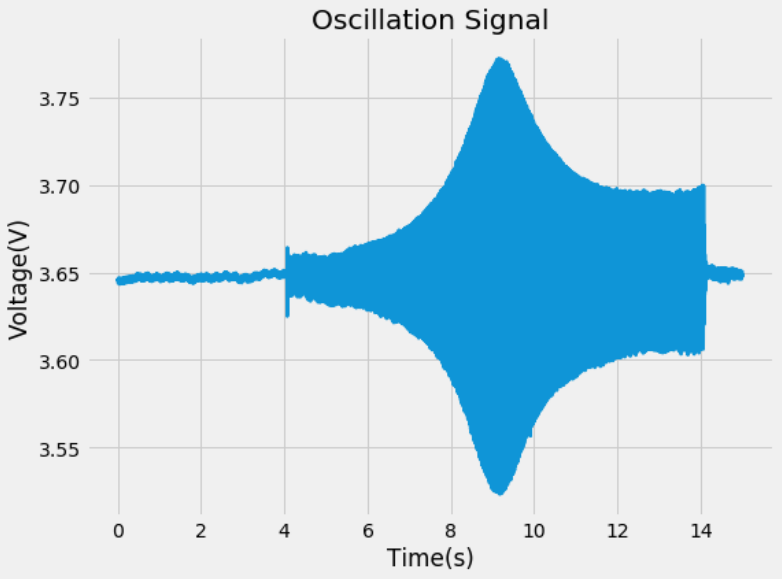


*Figure 15. A sample signal of the oscillation of a levitated droplet. The signal is filtered afterwards, as described in the materials and methods section in preparation for spectral analysis using a Fourier Transform.*

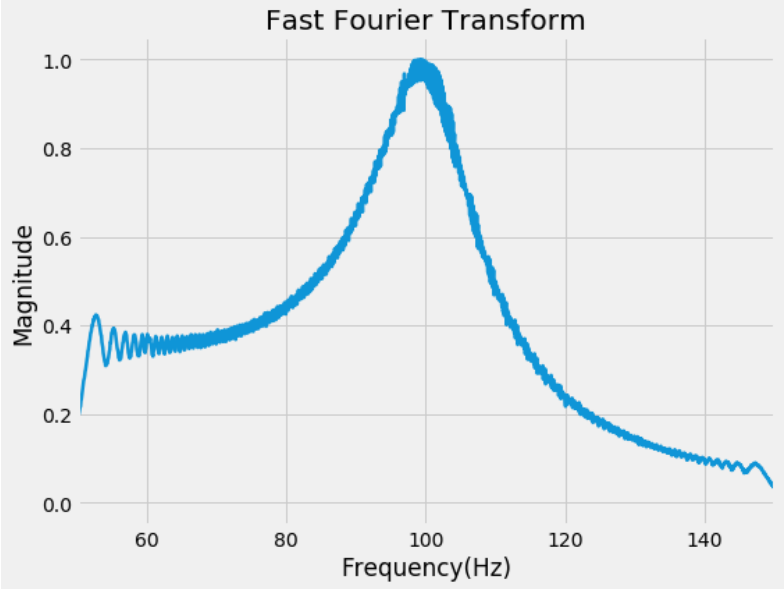


*Figure 16. A sample Fast Fourier Transform of the oscillation signal, revealing a broad resonance peak at around 100 Hz.*

The Fourier analysis also revealed the oscillation spectra of levitated drops with higher concentrations of polystyrene microparticles were more likely to exhibit an additional resonance peak, compared to the singular resonance peak of the lower concentrations of microparticles (control, .025M concentration, and .05M concentration). In 5 of 6 samples levitated, the .1M PSMP concentration droplet exhibited two resonance peaks at time zero. This was the case in only 2 of 7 samples with .05M PSMP concentration and were not seen in any of the samples for any of the other remaining groups.

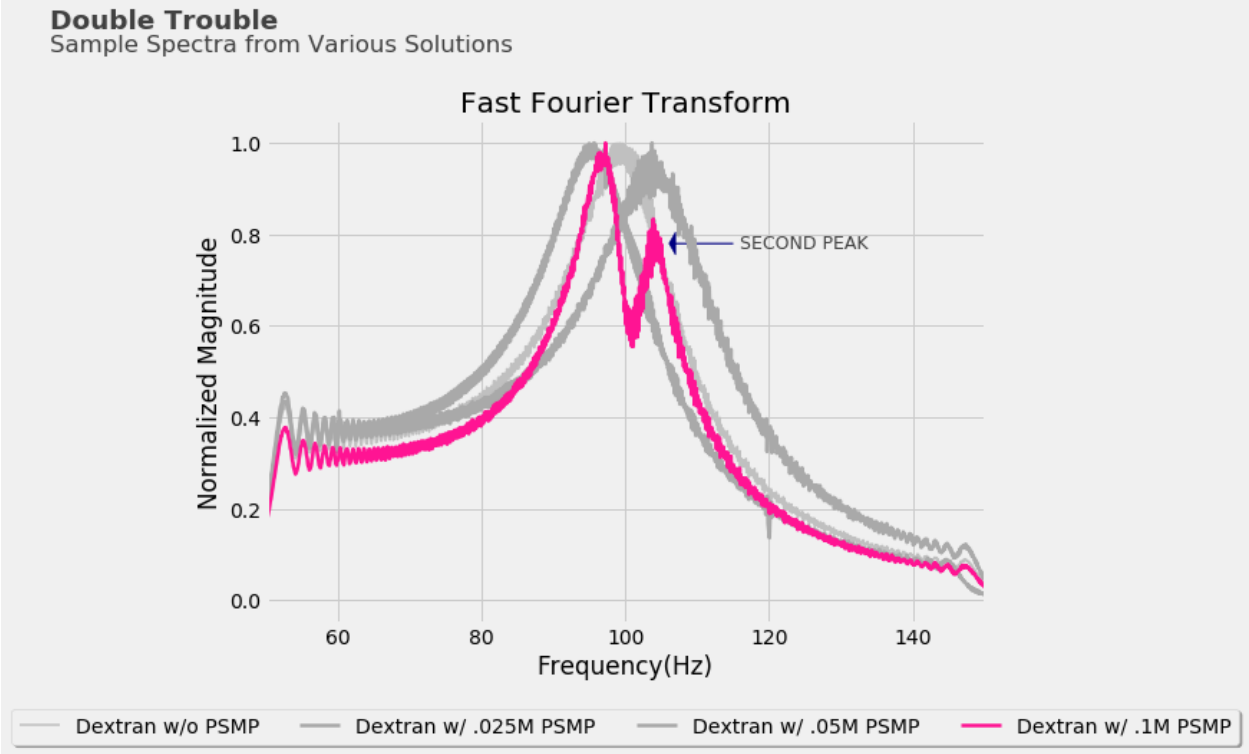


*Figure 17.Representative spectra from all tested control and experimental groups. Highlighted in pink is the spectrum of the experimental group with the highest concentration of polystyrene microparticles. It differs from the others as it features a second resonance.*

## II. Resonance Shifts

Furthermore, the frequency values of the resonant peaks each time point for each group were tracked. It was found for all groups, control and experimental, that with time, the resonant frequency shifts with time.

| **Group** | **Average Primary Resonance Frequency Shift (Hz/min)** |
|---|---|
| Control | 1.387 |
| +.025M PSMP | 1.521 |
| +.05M PSMP | 1.956 |
| +.1M PSMP | 1.632 |

*Table 4. The average resonance shift of the primary resonance peak of the spectra for each group..*

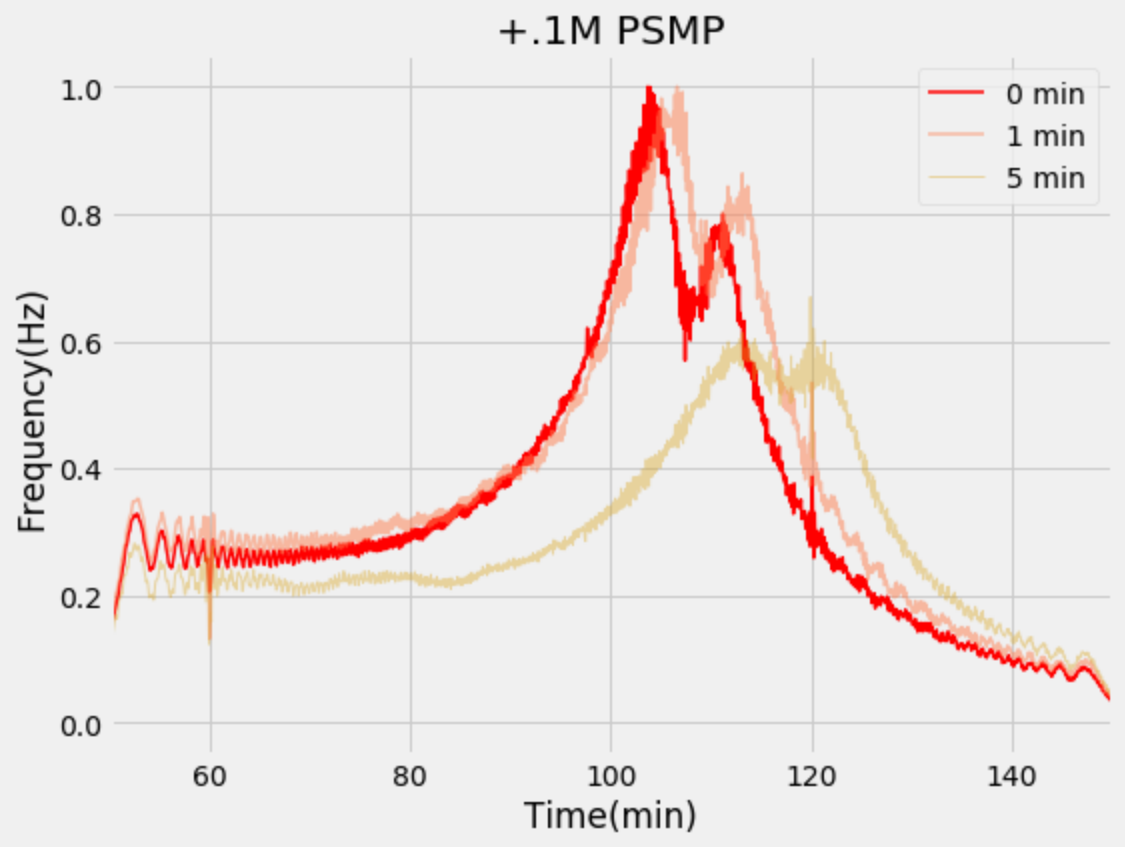


*Figure 18. Fast Fourier Transform from a tested sample droplet. With time, not only do the resonance frequencies shift, but the amplitude of the frequency shifts with it, broadening the resonance peaks and thus, dampening it.*

### III. Effect of Humidity

The effect on humidity on the resonance shift was explored. The primary resonance shifts were divided into groups based on the level of humidity recorded on the day of the experiments. Higher resonance shifting was found to be proportional to experimental environments of lower humidity.

| **Average Primary Resonance Shift** | | | |
|---|---|---|---|
| Group | Day 1 (49% Humidity) | Day 2 (33-38% Humidity) | Day 3 (27% Humidity) |
| Control | 1.204 (4 samples) | 1.631 (3 samples) | - |
| +.025M PSMP | 1.217 (4 samples) | 1.907 (3 samples) | - |
| +.05M PSMP | 1.687 (4 samples) | 2.314 (3 samples) | - |
| +.1M PSMP | 1.461 (3 samples) | - | 1.817 (3 samples) |

*Table 5. The average resonance shift of the primary resonance peak of the spectra, found during each day of experimentation. On each day, the humidity in the experimental laboratory differed.*

| **Average Secondary Resonance Shift** |
|---|

| Group | Day 1 (49% Humidity) | Day 2 (33-38% Humidity) | Day 3 (27% Humidity) |
|---|---|---|---|
| +.05M PSMP | - | 1.016 (2 samples)* | - |
| +.1M PSMP | 0.526 (2 samples)* | - | 2.132 (3 samples) |

*Table 6. The average resonance shift of the secondary resonance peak of the spectra, found during each day of experimentation. On each day, the humidity in the experimental laboratory differed. *Only two samples of the tested +.05M PSMP groups showed a secondary resonance peak. Only one of the samples of the tested +.1M PSMP groups did not show a secondary resonance peak.*

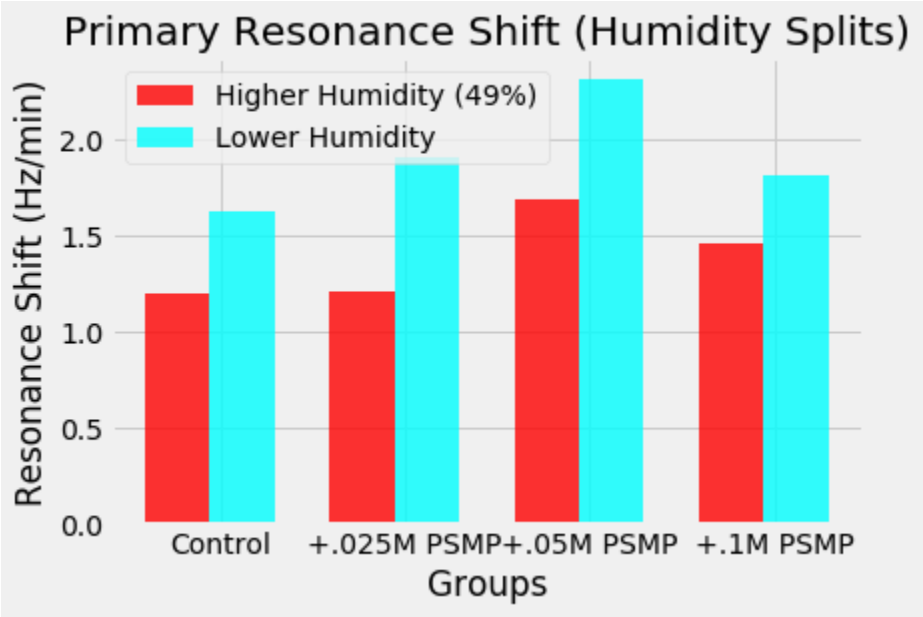


*Figure 19. Resonance shifts of the primary resonance peaks for the control and experimental groups, split by humidity. Higher humidity 49% for all groups, while lower humidity values were 38% for the control and the +.025M PSMP group, 33% for the +.05M PSMP group, and 27% for the +.1M PSMP group.*

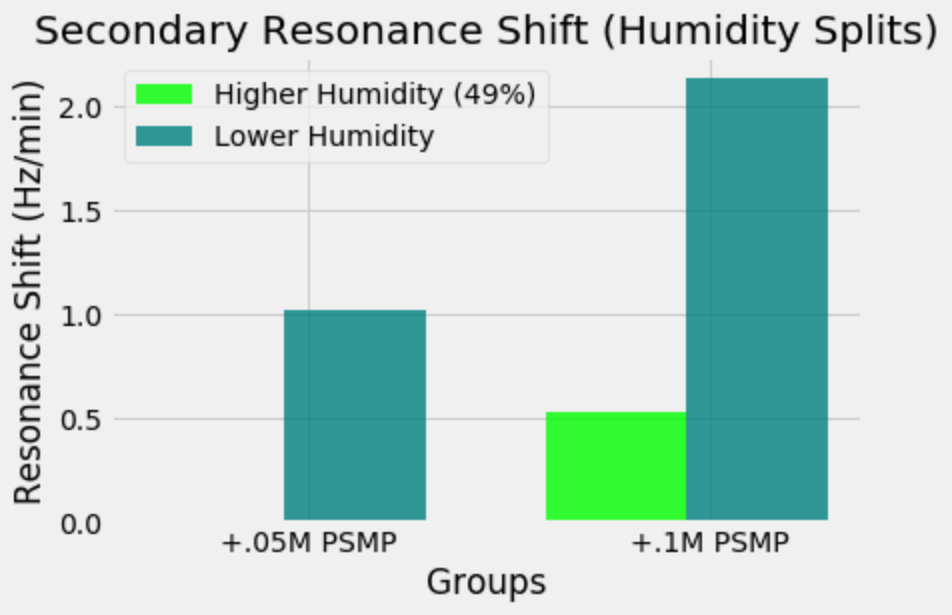


*Figure 20. Resonance shifts of the primary resonance peaks for the control and experimental groups, split by humidity. Higher humidity 49% for all groups, while lower humidity values were 33% for the +.05M PSMP group, and 27% for the .1M PSMP group.*

## IV. Quality Factor

The quality factor of each resonance peak was found using Equation 1, taking the frequency bandwidth at half-maximum amplitude. What is reported in each of the following graphs is the total quality factor of the spectra. Due to some spectra having multiple resonance peaks, the total quality factor of the system would be the sum of the quality factors from each of the peaks. It was found that the total quality factor of the spectra of samples with higher concentrations of microparticles (.05M and .1M PSMP) was greater than the spectra of samples with lower concentrations of microparticles (.025M and the control). It was also found with time, the total quality factor in the spectra decreased. However, the rate of decrease was not calculated due to discrepancies discovered in how the quality factor is calculated (this will be explained in the discussion section of this thesis).

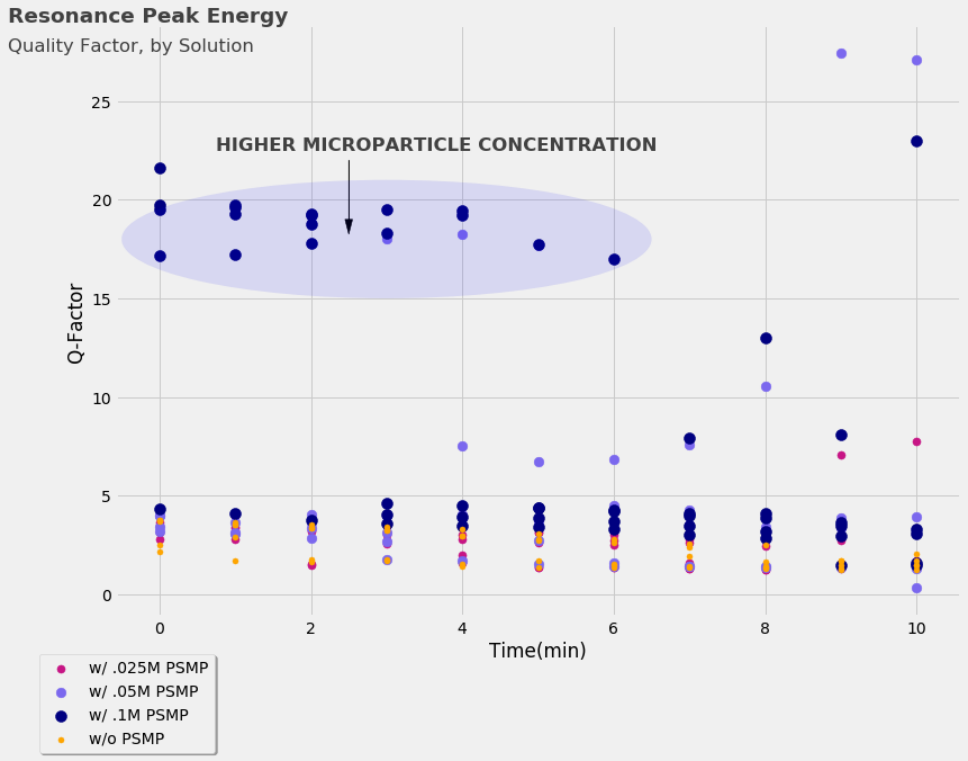


*Figure 21. Total quality factor for each sample levitated at each time point. While a general trend of high quality factor to low quality factor is observed, there are some outliers hanging around in the top right corner. These are likely miscalculations or misreading of the quality factor, and a solution on how to fix this problem in the code is divulged in the Discussion section.*

## V. Code Viability

The code written to achieve the purposes of this study have also been in the Biomedical Acoustics Laboratory to analyze the droplet oscillations of other fluids, other than aqueous dextran. Such fluids include whole blood, blood plasma, amniotic fluid, and other standard fluids. The same effects and patterns seen in the data obtained from the experiments of this study can also generally be seen in the data for the droplet oscillations of those fluids as well. Sample graphs showing these effects can be found in the Appendix.

## DISCUSSION AND CONCLUSION

From the results shown, there are spectral differences in fluid droplets and that these differences reveal themselves with different concentrations of polystyrene micro particles. However, the tribe effects of the microparticles on the resonances of the droplets are unknown due to potential overriding effects.

From the conclusions of Khismatullin and Nadim's study, it should be expected that oscillatory modes should decrease under conditions of low Reynolds number, or high viscosity. This was the case in the experiments done here—it was evident that the oscillation modes of the droplets decreased as the concentration of dextran in the aqueous dextran solution was raised. This is the reason that 5% aqueous dextran was chosen as the control, rather than water or aqueous solutions with lower dextran concentrations. Enough dextran in the solution and the oscillation modes were reduced to one. It was important the oscillation modes of the control were as few as possible, so that it would be easier to analyze the effects of the microparticles.

Aqueous dextran solutions also have negligible elasticity, as adding dextran to water does not result in a viscoelastic fluid. Therefore, according to Holt. et al's findings, the resonance modes of the aqueous dextran droplet should not have shifted with time. However, in these findings the resonance did shift with time.

Because experiments were performed on four different days, there were changes in the humidity of the laboratory during experiments. Because the humidity of the room was recorded prior to each experiment, the data could be split by humidity for further analysis. It was found that with lower humidity, there was an increase in resonance frequency shift per minute for all of the groups tested. It could be hypothesized that with lower humidity,

the more tendency for the drop to evaporate and thus cause changes in resonance frequency shift.

Because of this effect, it is difficult to quantify how much effect the microparticles have on the oscillation modes of the droplet. According to Khismatullin and Nadium, viscous droplets will show a new set of oscillatory modes if some elasticity is added to the model. These modes would be dependent on viscous and elastic stresses, rather than on surface tension (in cases of low viscosity fluids like water). Do the polystyrene microparticles add some elasticity to the droplet?

There is good reason to believe so. It was shown that in the droplets with .1 M of polystyrene microparticles, there was an additional resonance mode. This shows that there is a concentration threshold that if superseded, would illicit a secondary resonance peak. However, like with the primary resonance peak, it would shift and dissipate with time. If the conclusions of Holt et. al are to be accepted, the the shift in resonance should be due to elasticity. However, it is important to reiterate that how much of this shift is due to the elasticity of the microparticles cannot be accurately quantified.

As any levitated fluid droplet's oscillation signal would depreciate with time due to evaporation, solidification, or evaporation, it can become increasingly difficult to select the true resonance peaks of the signal (if any exist). With depreciating signal, the noise which went unfiltered may become more prominent and overlap with the true resonance of the signal. This is especially more likely in cases where the resonance has shifted and is closer to 120 Hz, which is a harmonic of the 60 Hz AC power line signal. This is problematic and leads to incorrect calculations of resonance frequency and quality factor, and corrections often must be made after the data is processed. This is time consuming and

inhibits the ability to continue on in the study. How might a new noise filter be designed to accommodate for this fact without employing an algorithm that would interfere with the bandpass filter already in place or interfere with the true signal values itself?

Noise peaks tend to be very sharp and narrow, while true resonances tend to be broader in shape. Because quality factor is dependent on the bandwidth of the resonance peak, a narrow bandwidth will lead to an abnormally high resonance peak. Therefore, in cases featuring peaks with significantly higher quality factors than its neighbors, those cases were investigated for noise peaks. Further iterations of the algorithm will be updated to include this observation, in the form of machine learning to investigate and add another layer of filter to the signal to account for abnormal quality factor trends.

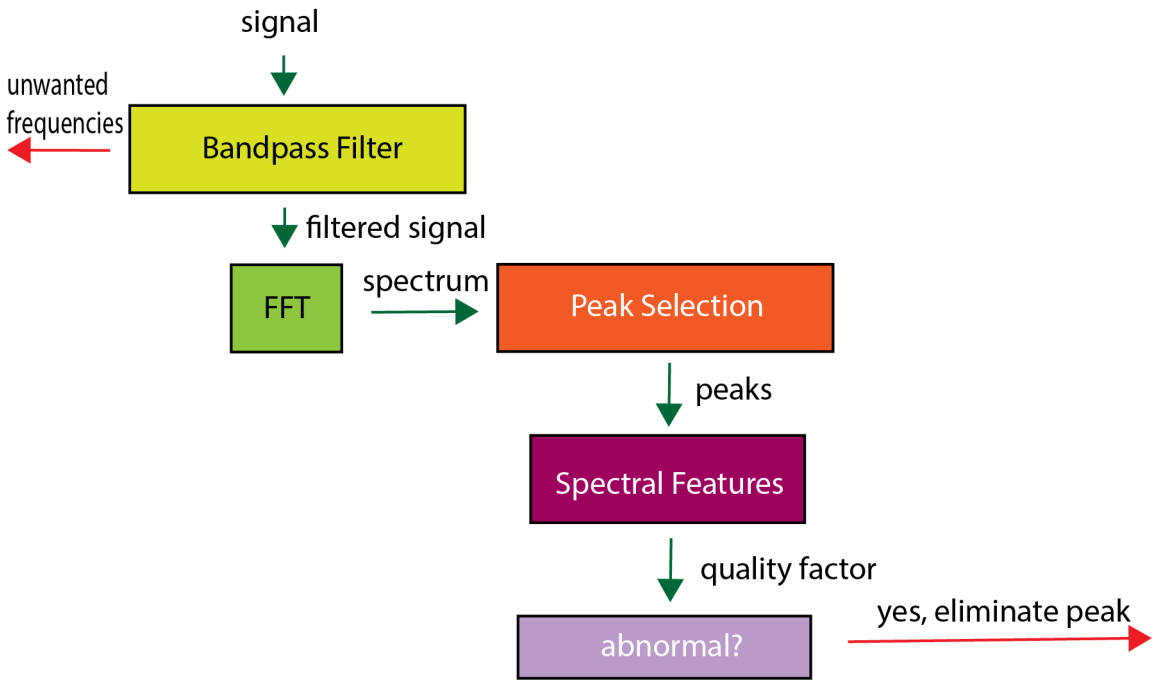


*Figure 22. In addition to the pseudocode diagram seen in the Materials and Methods section, this version is updated in response to the findings of this study. Abnormal quality factor values can be tracked back to its originator—noise peaks. Because of this observation, quality factor can be used a post-processing check to ensure that all of the right peaks are selected.*

There is reason to accept the premise that resonance features of the oscillations of fluid droplets are reflective of its composition. However, in order to reach a more definitive

conclusion on the factors which would make a phenomenon possible, further work must be done to isolate and study these variable factors more closely.

Such work includes tackling the issue of evaporation. With a smaller fluid droplet, the resonance of the fluid droplet will shift to the right (as a higher resonance frequency means that it takes more energy for the waves to move the particles of the droplet). A study can be performed to study whether there is significant difference in the resonances of fluid droplets of different size. A positive test from such a study would indicate the need to build a system that would enclose the droplet to prevent evaporation during experimentation. Such an enclosure would need to have low a attenuation coefficient, so that it would not affect the ability for ultrasound waves to pass through to the droplet.

Additionally, the current system does not account for the attenuation properties of the fluid droplet itself. How the droplet reflects sound back to the transducer is of useful analysis. Studies have been performed to study these reflected signals to distinguish cell types. It is this principle that allows for ultrasound images of internal structures of the body to be made due to the principle that different tissue structures attenuate sound differently. This additional information, coupled with studying the oscillations of the fluid droplet, may be helpful in determining more subtle differences in composition.

Even with more experiments and projects to be done, acoustic levitation is still a highly preferred method for analysis of small biological samples due to its ability to assess properties without fear of contaminating or changing sample properties via unintended contact.

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# APPENDIX

## I. Amplitude-Frequency Response of Sample Droplets

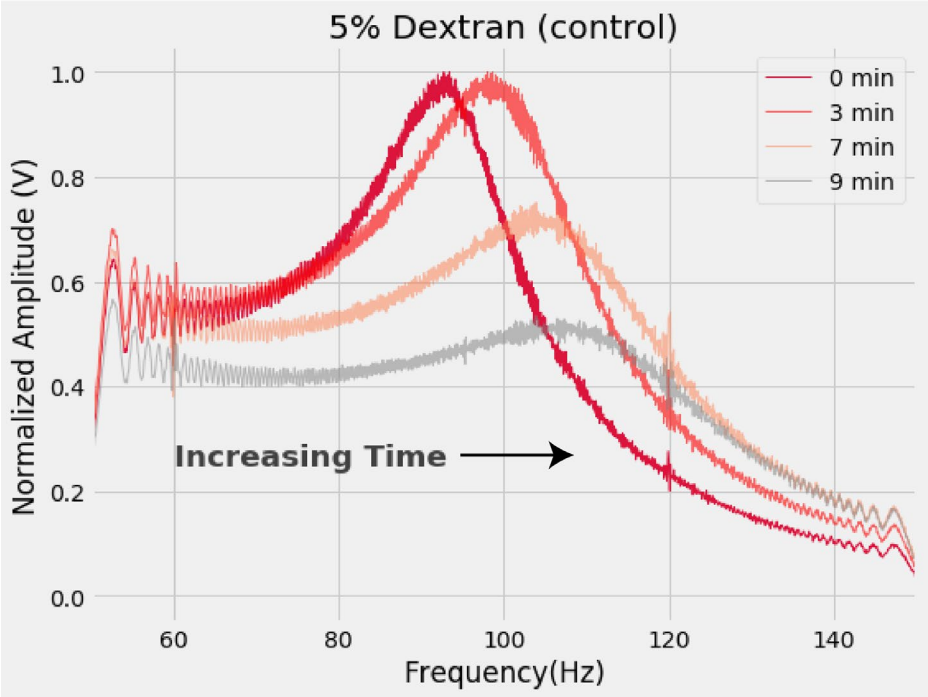


*Figure 23. A plot of oscillation spectra from the same aqueous dextran fluid droplet. It was seen that with time, the resonance frequency shifts and its amplitude dampens.*

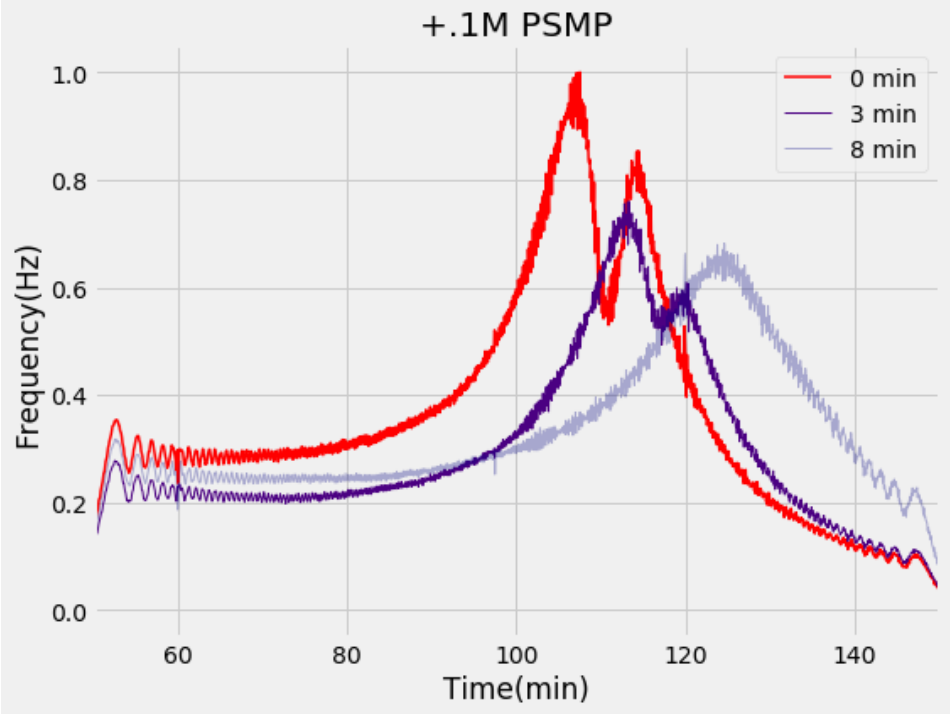


*Figure 24. The same shifting effect was seen even in oscillation spectra with two resonance peaks, rather than just one. The secondary peak diminished faster and was absorbed by the larger peak.*

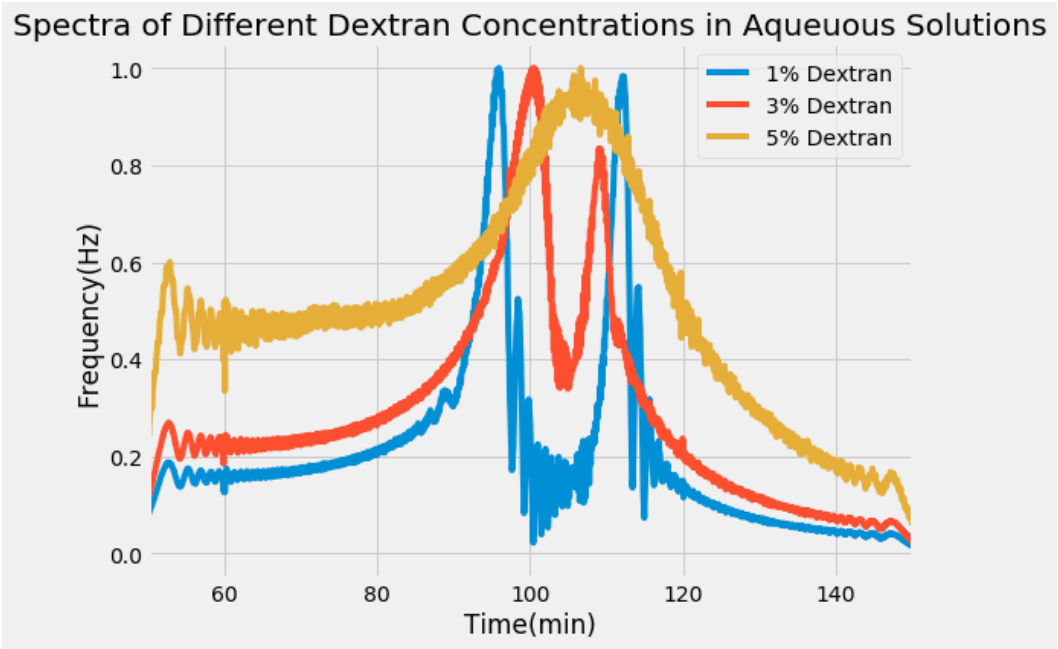


*Figure 25. To show the effect viscosity has on resonance modes, three different concentrations of aqueous dextran solutions were levitated. Raising the dextran concentration to 5%, thus increasing the velocity, lowered the number of oscillatory modes from two to one and provided a much more stable spectrum.*

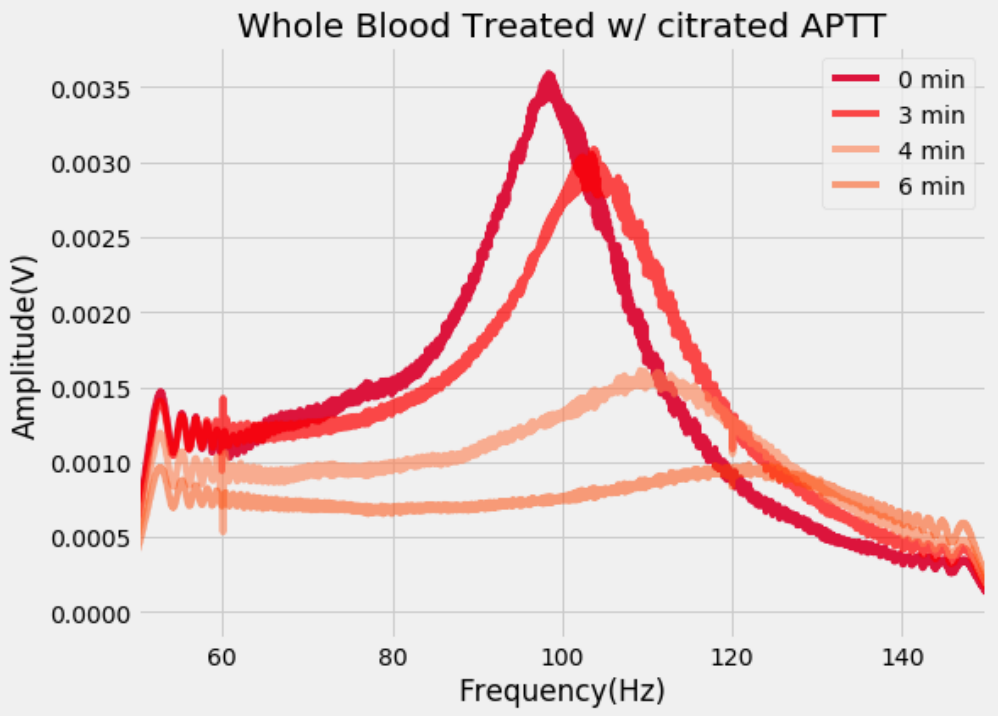


*Figure 26. The code in the appendix below has also been used to analyze oscillation spectra of other fluid droplets, like whole blood. The resonance shifts in blood are more prominent than in aqueous dextran due to blood's stronger behavior as a viscoelastic fluod.*

## II. MATLAB Code to Obtain Amplitude-Frequency Response

```
[source_files,source_dir]=uigetfile('*.csv','Select the csv
file.');
addpath(source_dir);
%% raw data processing
%importing the data
data = csvread(fullfile(source_dir, source_files),5,0);
raw_time = data(:,1);
raw_signal = data(:,2);

%sweep information - Need to find way to automatically detect this
(did
%it at least with filtering!)
startFreq = 150;
stopFreq = 100;


% %filter the raw signal (bandpass)
[real_time,real_signal, Fs] = humfilter(data);


%   Detailed explanation goes here
raw_time = data(:,1);
raw_signal = data(:,2);
```

```
Fs = 1/(mean(diff(raw_time))); %sampling rate
mmsignal = raw_signal - mean(raw_signal);
buttLoop = bandpass(mmsignal, [50 150], Fs);
real_signal = buttLoop;


%% taking the fft of the filtered signal
figure;
Y = fft(real_signal);
L = length(real_signal);
P2 = abs(Y/L);
P1 = P2(1:L/2+1);
P1(2:end-1) = 2*P1(2:end-1);
f = Fs*(0:(L/2))/L;


% FFT
plot(f,P1, 'DisplayName', 'FFT')
title('Fast Fourier Transform')
xlabel('f (Hz)')
ylabel('Normalized Amplitude (V)')
% xlim([0 150])


%peak analysis
[pks,locs,~,~]    =    findpeaks(Penv,    'MinPeakDistance',    70,
'MinPeakHeight', 1e-4, 'MinPeakProminence', 1e-2);

%% calculating Bandwidth Frequencies at Half-Maximum

halfMaxAmplitude = peak/2; %half of the maximum power;
vectorsize = size(envelope);
checkvector(1:vectorsize) = halfMaxAmplitude;

%sometimes the halfMax is not a point in the envelopepeaks array.
The below
%code finds the closest number in the envelopepeaks array to the
halfMax.

d = abs(envelope-halfMaxAmplitude); %subtracts the halfMax from
the envelope peaks function
[e, ix] = sort(d); %sorts them in ascending order and grabs indices
(ix)
%the following code goes through the entire HzScale and finds the
frequency
%before the PeakFrequency that is the closest to the halfMax

for j=1:length(ix)
    TestValue = f(ix(j));
    if count == 1
        if TestValue < Hz
```

```
            LeftFrequency = TestValue; %vector of all frequencies
before the PeakFrequency
            break
        end
    else
        if TestValue < Hz && TestValue > HzSET(count-1)
            LeftFrequency = TestValue; %vector of all frequencies
before the PeakFrequency
            break
        end
    end
end

%the following code goes through the entire HzScale and finds the
frequency past the PeakFrequency that is the closest to the
halfMax.

for l=1:length(ix)
    TestValue2 = f(ix(l));
    if count == length(HzSET)
        if TestValue2 > Hz
            RightFrequency = TestValue2;
            break
        end
    elseif count == 1
        if TestValue2 > Hz && TestValue2 < HzSET(count+1)
            RightFrequency = TestValue2;
            break
        end
    else
        if TestValue2 > Hz && TestValue2 < HzSET(count+1)
            RightFrequency = TestValue2;
            break
        end
    end
end
%% Quality Factor
Q = Hz/(RightFrequency-LeftFrequency); %quality factor
end
```