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Determining the mechanical properties of apoptotic cells using time resolved acoustic microscopy

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Ryerson University

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DETERMINING THE MECHANICAL PROPERTIES OF APOPTOTIC CELLS USING TIME RESOLVED ACOUSTIC MICROSCOPY

By

Eric Strohm

B.Sc. McMaster University, 1999

Hamilton, Ontario

A thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Science

in the Program of

Biomedical Physics

Toronto, Ontario, Canada, 2009

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Author's Declaration

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Abstract

Eric Strohm. "Determining the Mechanical properties of apoptotic cells using time resolved acoustic microscopy", M. Sc. Biomedical Physics, Ryerson University, Toronto, 2009

Time resolved acoustic microscopy uses ultra-high frequencies (over 100 MHz) to image cells with a resolution up to 1 μ m. Acoustic microscopy has been used since the 1970's for biological observations, however only recently have technological advances allowed the quantification of the physical properties of cells, such as the thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation using acoustic microscopy.

For the first time, the physical properties of MCF-7 cells treated with a chemotherapeutic agent to induce apoptosis were measured using a 375 MHz transducer. It was found the thickness and attenuation increased after apoptosis, while the speed of sound, acoustic impedance, density and bulk modulus were similar within measured error.

Acoustic backscatter measurements showed rapid morphological variations in cells undergoing apoptosis, which correlates to optical observations. This indicates extensive activity is occurring within the cell on a time scale of seconds during apoptosis.

Acknowledgements

I would like to sincerely thank my supervisor Dr. Michael Kolios for his support and confidence during this research. His excitement and enthusiasm about physics is not only motivating, but also highly contagious. I feel privileged to have worked with such a knowledgeable professional. I would also like to thank my supervisory committee, Dr. Carl Kumaradas, Dr. Jahan Tavakkoli and Dr. Greg Czarnota for their guidance during this research.

This work would not have been possible without the technical support provided by the lab; in particular, Dr. Min Rui and Anoja Giles for their expertise in cell cultures, and Arthur Worthington for his knowledge in ultrasound and technical issues. Dr. Eike Weiss was always available for technical discussions and I am grateful for his patience and feedback.

I would like to thank my mom, dad and sister, for their support and for always encouraging me to pursue my desires. Finally I would like to express my gratitude to my wife Bahar, who encouraged me to pursue a field I find exciting and rewarding. This work would not have been possible without her support.

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Chapter 1 Introduction

1.1 Motivation

The human body is composed of trillions of cells, all working together in harmony. Occasionally cells will mutate and start multiplying uncontrollably, creating a dense mass of tissue. These cancerous tumours can obstruct or impede the natural processes of nearby organs and tissue. The cancer cells may also metastasize to other locations of the body, creating new areas of tumour growth. Cancer is the second highest cause of death behind heart disease and will account for over 600,000 deaths in the United States in 2009, or 0.2% of the population [3]. Early detection and treatment are critical to improving the survival rate from cancer.

Methods for tumour detection include visual inspection, testing for genetic markers, MRI, X-ray, CT-scan, and clinical ultrasound [4, 5]. Typical cancer treatments consist of removal or destruction of the tumour (through surgery or ablation), chemotherapy, radiation therapy, immunotherapy, hormone therapy or a combination of the above [6]. Newer treatment methods gaining FDA approval include high intensity focused ultrasound, radio-frequency ablation and laser interstitial therapy [7, 8]. Since current methods of tumour

imaging are either cost-prohibitive or invasive, a method to monitor the response of a tumour during treatment over the course of several weeks is difficult to implement.

1.2 Cell Death

Apoptosis, or programmed cell death, is a controlled method for cells to die with minimal disruption to surrounding tissue [9]. Apoptosis is used to maintain homeostasis in the body, to keep the number of cells relatively constant. A cell may mutate and lose the ability to undergo apoptosis, and grow out of control resulting in a tumour. Chemotherapy treatments concentrate on killing cancer cells and restoring homeostasis. The tumour will eventually shrink in response to the chemotherapy treatment. Apoptosis can be triggered when a cell is diseased or damaged, or through other signalling methods including hormones and toxins.

Necrosis is another type cell death, typically due acute injury [10]. Cell death from necrosis is uncontrolled and usually induces an inflammatory response by expelling the toxic intracellular contents into the surrounding tissue. A strong inflammatory response can lead to an increased morbidity and mortality, in contrast to the controlled method by apoptosis.

1.3 Ultrasound

High frequency sound waves have been used since the mid-1900's for medical diagnostic imaging [11]. Ultrasound is non-invasive, low risk and inexpensive compared to other imaging modalities, making it an ideal method of clinical imaging. Ultrasound could be used to monitor the efficacy of a treatment, during the course of a treatment. Monitoring the

tumour response would potentially allow customization of the treatment to the individual patient. If the treatment is found to be ineffective, an alternate treatment could be formulated.

Figure 1-1: Resolution as a function of frequency for two transducers with different f-numbers. Clinical ultrasound (1-15 MHz) is capable of deep penetration into the body, but lacks the ability to resolve fine details. Acoustic microscopy has excellent resolution, however, penetration depth is limited.

Figure 1-1 shows how the ultrasound resolution improves with increasing frequency [12]. As the frequency increases, the resolution increases but comes at a cost of penetration depth. Penetration depth depends on the attenuation of the propagation medium, and the attenuation increases with frequency. Clinical ultrasound (1-15 MHz) is able to penetrate 5-15 cm depending on the tissue type, and is ideal for deep body imaging, however it lacks resolution to detect fine details [13]. High frequency ultrasound (20-80 MHz) has good resolution, but is unable to penetrate more than a few cm into the body, making it useful only

for surface imaging. Acoustic microscopy (100+ MHz) has excellent resolution, up to 1 μm at 1 GHz, but can penetrate only a few cell thicknesses and is unsuitable for clinical imaging.

1.4 High Frequency Studies

Recent studies using high frequency ultrasound (20-60 MHz) observed an increase in the ultrasound backscatter intensity for cells treated with chemotherapeutic drugs to induce apoptosis compared to untreated cells, and also changes in the frequency dependence of the ultrasound scattering [14, 15, 114]. These studies used cell pellets created by centrifuging cells into a dense arrangement of cells to mimic bulk tumour tissue. In one experiment, the cell pellet contained three layers; the middle layer was composed of cells treated and undergoing apoptosis, and untreated cells in the top and bottom layers. As shown in Figure 1-2, an increase in the ultrasound backscatter intensity was observed for the treated layer compared to the untreated layers [1, 2]. In addition, clear delineation of the treated and untreated cell areas were observed. Note that at the frequencies used in these studies, individual cells cannot be resolved. What is observed is the speckle pattern from sub-cellular components.

It was hypothesized the structural changes within the apoptotic cells were responsible for the increase in backscatter observed [16]. However the source of increased backscatter could also be due changes in the cellular spatial rearrangement [17]. The source of increased backscatter intensity is unknown and cannot be determined using HF ultrasound alone. Additional studies must be performed to determine the source of increased backscatter. Critical to this is knowledge of cell properties at scales close to the wavelengths used such as

those in acoustic microscopy.

Figure 1-2: Cells at 20 MHz (A) and 40 MHz (B) show an increase in ultrasound backscatter intensity with treated cells. In these studies, a cell pellet was created with cells treated to induce apoptosis in the middle, and untreated cells in the top and bottom layers (Duplicated from [2]).

1.5 Measuring the Properties of Cells

A key to understanding the observations with HF ultrasound is knowing the properties of cells, such as the density, sound velocity, acoustic impedance, bulk modulus, and attenuation interact with ultrasound. These properties can change through various physiological processes such as apoptosis, mitosis, locomotion, and adhesion [18] and how they change as a function of time through these processes is poorly understood.

Various techniques have been used to measure these mechanical properties, including embedded particle tracking [19], laser tracking microrheology [20], magnetic twisting cytometry [21], micropipette aspiration [22], microneedles [23], optical [24] and magnetic tweezers [25], and atomic force microscopy [26]. These techniques are invasive, requiring staining and/or a cellular response to an external stimulus [27].

While the above methods can be used to measure cells in a steady state, it can be difficult or impossible to measure cells during rapid processes, such as apoptosis and mitosis. Moreover, external stimuli can disturb the process, and therefore alter the natural physiological response of a cell. In addition, some techniques average the properties of the entire cell and are unable to restrict measurements to specific regions, such as the nucleus and organelles.

1.6 Acoustic Microscopy

Acoustic microscopy was developed in the early 1970's by Lemons and Quate [28], and one of the earliest applications was to examine biological material using ultra-high frequencies (over 100 MHz) [29]. While unsuitable for bulk tissue investigations due to the large attenuation at higher frequencies, acoustic microscopy has the resolution and capabilities to resolve individual cells and organelles as they undergo various biological processes [30]. It is non-invasive, and able to measure live cells without staining or fixation, a major advantage over other measurement techniques. The scattered ultrasound from cellular microstructures would provide valuable information as to the source of the backscatter within the cell, and help understand previous observations at high frequencies (10-60 MHz). Acoustic microscopy is also capable of quantitative measurements of some of the physical properties of cells of interest to ultrasound imaging, such as the sound velocity, acoustic impedance, density, bulk modulus and attenuation. These parameters can be used in computer modeling and simulations to help understand the apoptosis process and imaging of bulk tissue at high frequencies [31, 32].

Early acoustic microscopes were able to create high contrast images dependent on the mechanical properties of cells, however, quantitative analysis was limited due to technology limitations. Different methods of measuring the mechanical properties of cells include the interference fringe method in which long ultrasound pulses were used, measuring the signal as a function of position ($V(z)$) or frequency ($V(f)$), and time resolved methods.

The interference fringe method can be used to obtain the mechanical properties in a sample using narrowband pulse bursts. Waves reflected from the surface of a sample and the substrate will interfere, forming interference patterns on the sample image [33]. These fringes represent equal path lengths in the sample, and can be used to estimate the thickness, sound velocity and acoustic impedance in the sample. While these measurements can be done quickly, they suffer from poor resolution and the measurements are limited to regions showing visible fringe separation. In addition, this method assumes minimal variations in the density and composition of a cell, which is not always correct.

The thickness, sound velocity and attenuation in endothelium cells of xenopus tadpole hearts was calculated using the interference fringe method using a transducer at 1 GHz [34]. The sound velocity and attenuation measurements suffered from low precision, and measurements were impossible around the nucleus. An advancement in the interference fringe method, increasing accuracy, was used to determine the sound velocity in human aortic smooth muscle cells at 1571 m/s [35].

Later techniques used $V(z)$ methods, where the signal is measured as a function of axial position thorough the sample. This method requires images recorded at different focal

positions through the sample. Gathering enough images for a quantitative analysis is time consuming, and this method is generally reserved for measurements of fixed specimens only [12].

Measuring the signal as a function of frequency, known as $V(f)$ curves, can also be used to determine the mechanical properties. The thickness, sound velocity and attenuation in a sample can be obtained by varying the frequency during a measurement [36]. Endothelium cells of xenopus tadpole hearts were characterized using $V(f)$ curves to determine the thickness, sound velocity and attenuation [36]. The sound velocity ranged from 1.5 to 1.7 m/s, and was generally higher around the nucleus. The attenuation ranged from 0.1 to 0.15 (no units given). This method has significant limitations, as the measurements are lengthy, requires both the phase and amplitude of the signal, and measurements at multiple frequencies [37]. Transducers are expensive and difficult to fabricate, and thus a small selection of transducers are available during experimentation which limits the frequency range available.

Recent advancements in computers and technology enabled recording of the RF-pulse as a function of time at high sampling frequencies (GHz range). Short pulses combined with an increased signal to noise ratio (SNR) from post-processing methods allowed for time resolved measurements, where the backscatter echoes from the sample and substrate can be resolved separately. This is a significant advancement over other acoustic microscopy methods in that the mechanical properties such as the thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation can be directly obtained with a single measurement [37].

Recent experiments using time resolved methods found the sound velocity of HeLa cells was 1550 m/s using 860 MHz, and the attenuation to be 1737 dB/cm [38]. In a separate study, HeLa cells were found to have a sound velocity of 1535 ± 34 m/s using 860 MHz [39]. A study measuring f-actin and cytosol in chicken cardiomyocyte using 1 GHz found a bulk modulus of 2.35-2.47 GPa and 2.25-2.28 GPa, respectively [40]. Acoustic microscopy using narrowband pulse bursts determined the attenuation increased for HeLa cells when they underwent apoptosis, however this was based on a qualitative assessment, no quantitative analysis was possible [41].

Biological examination presents some difficulty over isotropic and homogenous solids, as they are complex organisms made up of many components which can be time variant. In typical acoustic microscope systems, it is not possible to optically view the sample when measurements are made, therefore it is impossible to know exactly what has been imaged. The acoustic microscope used in this research has the capability of simultaneous optical and acoustic measurements, a distinct advantage over other microscope systems.

1.7 Apoptosis

Cells are complex living organisms, and are the building blocks of multi-cellular organisms [115]. Human cells are typically 5-20 μm in diameter. The majority of the cell is composed of cytoplasm, and cells are 60-90% fluid [42]. The rest of the composition is made up of proteins and organelles, such as the nucleus, mitochondria, vesicles, lysosomes, endoplasmic reticulum, and golgi apparatus [42].

Cancer cells are a collection of cells that continue dividing, disrupting normal

homeostasis [43]. These cells have mutated to lose the ability to undergo apoptosis. Left untreated, cancer cells will continue to duplicate, creating a large dense mass which may eventually disrupt normal body functions. Additionally, some cells may metastasize by breaking loose from the tumour and continue growing in a separate location. Inducing apoptosis in cancer cells is critical in chemotherapy treatments.

Apoptosis, or programmed cell death, is a controlled method of cellular disassembly designed to minimize disruption and damage to surrounding tissue [9, 44]. It is thought to be an integral part in the response of tumors to chemotherapeutic treatment [45, 46], but is also involved in other biological functions such as homeostasis [47, 48], embryonic development [49] and lymphocyte development [50].

Deregulation of apoptosis is present in a variety of diseases, such as Alzheimer's and AIDS, and inhibition of apoptosis can result in autoimmune disorders and tumours [51]. Cancerous tumours grow due to a mutagenic ability that prevents apoptosis from occurring. Therefore the study of apoptosis is critical to understanding cancer growth and formulating treatments for this disease [52].

Cells undergo apoptosis in response to specific triggers such as physical damage, ionizing radiation, immune response, and chemotherapy [53-56]. There is a delay between the exposure to the trigger and observation of apoptotic processes, which is variable and depends on the trigger type, environment and cell type [57].

In contrast to apoptosis, necrosis is a method of cell death that can be detrimental to surrounding tissue. The uncontrolled method of cellular destruction releases toxic intra-

cellular contents into the surrounding area, causing an inflammatory response and disruption of normal biological activities [10].

The first stage of apoptosis involves the activation of caspases, proteins which digest structural proteins in the cytoplasm and DNA degradation [55]. Cell shrinkage occurs, and the nuclear material condenses. The chromatin becomes pyknotic and condenses against the nuclear membrane. The DNA material then fragments, and the nucleus breaks into separate bodies within the cell. Membrane blebbing of the cell occurs, which may contain nuclear material [58]. Eventually the apoptotic cell may break into apoptotic bodies, which can then be engulfed by phagocytes. The entire process of apoptosis can take place quickly, from first signs of blebbing to removal of the apoptotic bodies in 30 minutes [10]. The early stages of apoptosis are reversible, allowing a cell to stop the process if the environmental conditions change [59]. Figure 1-3 shows a typical cell before and after morphological changes are observed during apoptosis.

Detection of apoptosis in cell cultures is possible by labeling the cell with a fluorescent stain that binds to specific markers during various stages of apoptosis. A popular method of apoptosis detection is by labeling fragmented DNA with terminal deoxynucleotidyl transferase dUTP (the TUNEL method) [60]. Hoechst 33342 is also used to label DNA, however, it cannot be used to differentiate between apoptotic and necrotic cells unless used in conjunction with propidium iodide [50]. Another method of apoptosis detection is using Annexin V to label phosphatidylserine, a phospholipid that becomes exposed to the membrane during apoptosis and is a signal for phagocytosis [61].

Figure 1-3: An optical image of a typical apoptotic cell before (left) and after (right) rounding up during apoptosis was observed. The cell on the left shows signs of membrane blebbing around the edge of the cell, a typical feature observed during apoptosis.

Current methods of apoptosis detection in bulk tissue include PET/SPECT [62-68], lipid proton MR spectroscopy [69-71], and diffusion weighted MRI [72-75]. These methods are invasive, require contrast enhancers or markers, radiation exposure and/or are expensive, which limit the number of tests that can be performed. Detection of apoptosis via ultrasound would provide an inexpensive and safe method of monitoring treatments.

There are many stages in apoptosis; this research concentrates on measuring the properties before and after irreversible rounding up during apoptosis occurs, where the morphological changes are easily observable without staining (Figure 1-3).

1.8 Hypothesis and Objectives

The objectives of this research is to validate the acoustic microscopy technique by measuring the mechanical properties of a specimen with known properties, then use this technique to measure the properties of cells undergoing apoptosis. We hypothesize time

resolved acoustic microscopy can be used to measure the mechanical properties of cells, such as the sound velocity, acoustic impedance, density, bulk modulus and attenuation during apoptosis. The goal is to determine how the properties change as the cells undergo apoptosis, which will give an indication of the structural changes that are occurring within the cell.

Chapter 2 Ultrasound Theory

2.1 Ultrasound Imaging

Ultrasound waves are high frequency sound waves inaudible to the human ear, or greater than 20 kHz. The propagation of sound waves in a homogeneous medium is governed by the wave equation

$$\nabla^2 u - \frac{1}{c^2} \frac{\partial^2 u}{\partial t^2} = 0 \quad 2-1$$

where u is the displacement, t is time, c is the phase velocity, and ∇ is the Laplace operator [76]. The equation of a traveling wave

$$u = u_0 e^{i(\omega t - kr)} \quad 2-2$$

is a solution to the wave equation with phase velocity

$$c = \frac{\omega}{k}, \quad 2-3$$

where ω is the angular frequency and k is the wave number. Waves can travel longitudinally (with the direction of propagation) or transverse (perpendicular to the direction of propagation).

2.1.1 Elastic Constants

An elastic modulus is related to the ability of an object to deform when a force is applied and is the ratio of the stress to the strain [77]. Stress is the force per area causing the deformation (typical units are GPa) and strain is the change in shape from the stress, in relation to the original shape. An object has many elastic moduli which depend on how and where the stress and strain are applied. Typical elastic moduli used to describe an object are the bulk modulus, Young's modulus, Poisson's ratio, shear (S-wave) modulus, primary wave (P-wave) modulus and Lamé's first parameter. The elastic constant C depend on the density ρ and the phase speed c through the object

$$C_{ij} = \rho c^2, \quad 2-4$$

where the subscripts ij are used to indicate the elastic constant direction in regards to the direction and type of wave. For isotropic homogenous solids, only two elastic constants are required to describe all the elastic moduli due to symmetry; any elastic moduli can be derived from the two known parameters [77].

Two elastic moduli that can be measured directly using ultrasound are the longitudinal modulus, or the p-wave modulus M

$$C_{11} = M = \rho c_l^2, \quad 2-5$$

and the shear modulus μ

$$C_{44} = \mu = \rho c_s^2, \quad 2-6$$

where the subscripts l and s denote the longitudinal and shear velocities, respectively. The

bulk modulus is the ability of an object to resist uniform compression when a pressure is applied, and is the inverse of compressibility. It is defined as

$$B = -V \frac{\partial P}{\partial V} = C_{11} - \frac{4}{3}C_{44}, \quad 2-7$$

where P is the pressure and V is the volume. Other common elastic moduli, derived from the shear and longitudinal moduli, are listed in Table 2-1.

Elastic Modulus	Description	Equation
Bulk Modulus	Resistance to uniform compression	$B = C_{11} - \frac{4}{3}C_{44}$
Young's Modulus	$\frac{\text{longitudinal stress}}{\text{longitudinal strain}}$	$G = C_{44} \frac{3C_{11} - 4C_{44}}{C_{11} - C_{44}}$
Shear Modulus	shear stress / shear strain	$\mu = C_{44}$
Poisson's Ratio	transverse strain / axial strain	$\sigma = \frac{C_{11} - 2C_{44}}{2C_{11} - 2C_{44}}$
P-Wave Modulus	axial stress / axial strain	$M = C_{11}$
Lamé's first parameter	Theoretical elastic parameter	$\lambda = C_{11} - 2C_{44}$

Table 2-1: A summary of commonly used elastic moduli for isotropic, homogenous media. The equations for each elastic modulus are written in terms of the two elastic moduli easily measurable using ultrasound, the shear modulus $C_{44}=\mu$ and the longitudinal (P-wave) modulus $C_{11}=M$.

In fluids, shear waves are greatly attenuated and can be neglected. Therefore the shear modulus $\mu = 0$. Consequently the bulk modulus can then be simplified to

$$B = C_{11} = \rho c^2. \quad 2-8$$

Therefore if the speed of sound and density of a fluid is known, the bulk modulus can be easily determined. Since fluids cannot support shear waves, by definition the Young's

modulus is zero, Poisson's ratio is 0.50 and Lamé's first parameter is equal to the bulk modulus. Using equation 2-4, the wave equation can be rewritten using the bulk modulus and density of the medium

$$B \nabla^2 u - \rho \frac{\partial^2 u}{\partial t^2} = 0. \quad 2-9$$

This equation demonstrates that the bulk modulus and the density of an object have a direct impact on how a wave propagates within the object. Cells are composed of 60-90% water [42], and modeling simulations have shown that cells with a nucleus to cell volume ratio of 0.33 are best represented as a fluid structure [78]. Therefore neglecting the shear component is a valid approximation in this research, as the main object of study are cells.

2.1.2 Scattering and Reflection

When ultrasound waves are incident on an interface between two materials, such as water and the membrane of a cell, a portion of the wave will be reflected from the cell, and a portion will be transmitted through it. Every material has a characteristic acoustic impedance which defines the reflection and transmission properties [76]. The acoustic impedance is defined as the product of the density ρ and speed of sound c within that object,

$$Z = \rho c, \quad 2-10$$

and has units $\text{kg/m}^2\text{s}$, or MRayls. For plane waves normally incident on an interface, the reflected R and transmitted T components can be calculated using

$$R = \frac{Z_2 - Z_1}{Z_2 + Z_1}, \quad 2-11$$

$$T = \frac{2Z_2}{Z_2 + Z_1}, \quad 2-12$$

where Z_2 and Z_1 are the acoustic impedances of the two materials at the interface [76]. For waves incident on an interface at a non-normal angle, Snell's law can be used to determine the change in the sound velocity as a wave passes from one material into another. the waves will be refracted according to Snell's law

$$\frac{\sin \theta_1}{\sin \theta_2} = \frac{c_1}{c_2}, \quad 2-13$$

where θ_1 and θ_2 are the angles of incidence, and c_1 and c_2 the velocities of the incident and refracted wave, respectively [12]. For a wave propagation normal to an interface, the frequency and the normal component of k must be conserved across the fluid-solid interface [79]. We can rewrite the normal component of the acoustic impedance at the boundary as

$$Z = \frac{\rho c}{\cos \theta}, \quad 2-14$$

and therefore the reflection and transmission coefficients can be rewritten as

$$R(\theta) = \frac{Z_2 \cos \theta_i - Z_1 \cos \theta_t}{Z_2 \cos \theta_i + Z_1 \cos \theta_t}, \quad 2-15$$

$$T(\theta) = \frac{2Z_2 \cos \theta_i}{Z_2 \cos \theta_i + Z_1 \cos \theta_t}. \quad 2-16$$

When a wave propagating in a fluid is incident on a solid, longitudinal and shear waves are created and must be accounted for in the wave analysis [80]. The velocity of longitudinal and shear waves are different, and therefore the acoustic impedance of these waves must be

defined separately, as

$$Z_0 = \frac{\rho_0 c_0}{\cos \theta}, \quad Z_l = \frac{\rho_l c_l}{\cos \theta_l}, \quad Z_s = \frac{\rho_s c_s}{\cos \theta_s}, \quad 2-17$$

where the subscript 0 denotes the coupling fluid, and the subscripts l and s denote the longitudinal and shear components in the solid substrate. Using these acoustic impedances, and conservation of the wave vector k at the fluid-boundary interface, the reflection and transmission coefficients become

$$R(\theta) = \frac{Z_2 \cos^2 2\theta_s + Z_s \sin^2 2\theta_s - Z}{Z_2 \cos^2 2\theta_s + Z_s \sin^2 2\theta_s + Z}, \quad 2-18$$

$$T_l(\theta) = \frac{2Z_l \cos 2\theta_s}{Z_2 \cos^2 2\theta_s + Z_s \sin^2 2\theta_s - Z}, \quad 2-19$$

$$T_s(\theta) = \frac{-2Z_s \sin 2\theta_s}{Z_2 \cos^2 2\theta_s + Z_s \sin^2 2\theta_s - Z}, \quad 2-20$$

where $T_l(\theta)$ is the longitudinal transmission coefficient and $T_s(\theta)$ is the shear transmission coefficient [12].

In addition to longitudinal and shear waves, another type of wave generated at fluid-solid interfaces are Rayleigh waves, which are a superposition of shear and longitudinal waves [81]. These waves will be discussed further in section 2.1.6.

2.1.3 Signal Measurement

Figure 2-1 shows a typical acoustic microscope system, with a transducer positioned so the ultrasound is focused onto the sample. The transducer consists of a piezoelectric

material and sapphire lens, as shown in Figure 2-1. The piezoelectric material will vibrate in response to an applied voltage, creating plane waves that travel down the buffer rod in the transducer. The plane waves are focused onto the sample by the spherical cavity. Waves from the sample and substrate are reflected back towards the transducer and are converted into plane waves by the cavity, which are then converted into a signal as a function of time by the piezoelectric material.

Figure 2-1: Typical transducer construction showing the transducer focused onto a sample to be measured.

The initial pulse emitted from the transducer, $s_0(t)$, will be reflected from a sample back towards the transducer. The reflected signal $s(t)$ recorded at the piezo will be

$$s(t) = Rs_0(t) \otimes g(t, z) \quad 2-21$$

where R is the reflection coefficient, $g(t, z)$ describes the wave distortion due to the lens, defocusing and attenuation and $\otimes$ is the convolution operator [30]. Generally z is the

defocus position, where $z = 0$ is with focus at the sample and negative values indicate the focus is below the sample. The signal $s(t)$ received at the transducer is converted into a voltage by the piezoelectric, and is the total contribution of reflected waves from the sample at a distance z over the entire transducer aperture half-angle θ_0 .

The waves will experience a distortion as they pass through the lens, from the lens into the coupling fluid and vice versa. The modified waves can be described by a pupil function, which defines the sensitivity of the lens as a function of aperture angle [82]. Lens distortions and aberrations may be accounted for in the pupil function [12]. It can be roughly approximated by using a rect function, by setting it equal to one within the lens aperture and zero outside

$$P(\theta) = \begin{cases} 1, & \theta \leq \theta_0 \\ 0, & \theta > \theta_0 \end{cases} \quad 2-22$$

where θ_0 is the half-aperture angle of the lens. However this function is discontinuous at the aperture angle θ_0 . A commonly used continuously differentiable pupil function that resembles the true behaviour of a lens is [83]

$$P(\theta) = \begin{cases} \frac{1}{2} \left[1 + \cos \left(\pi \frac{\cos \theta - 1}{\cos \theta_0 - 1} \right) \right], & \theta \leq \theta_0 \\ 0, & \theta > \theta_0 \end{cases} \quad 2-23$$

Phase differences are introduced which are dependent on θ , however these distortions can be minimized by careful selection of the lens materials which will be discussed further in section 3.2.

The final measured signal depends on the pupil function $P(\theta)$, reflectance function $R(\theta)$ and the initial emitted signal amplitude V_0 . The contribution of all waves reaching the transducer lens can be found by integrating over the aperture angle θ_0

$$s(t) = V_0 \int_0^{\theta_0} P(\theta) R(\theta) e^{-i2zk \cos \theta} \sin \theta \cos \theta d\theta, \quad 2-24$$

where $e^{-2zk \cos \theta}$ is the phase delay associated with defocusing in the z -direction, and $\sin \theta$ and $\cos \theta$ are terms dependent on the lens curvature [30]. The Fourier transform of equation 2-24 can be used to determine the reflectance function, and then the mechanical properties of the sample if the pupil function can be accurately determined [84]. However this method assumes the attenuation in the coupling fluid is relatively small and both the phase and amplitude of the signal is recorded [12]. This method allows accurate determination of the mechanical properties throughout the sample, but is computationally complex and requires very long acquisition times which may not be suitable for living cells.

2.1.4 Time Resolved Quantitative Measurements

Time resolved acoustic microscopy uses short highly focused ultrasound pulses to determine the mechanical properties of a sample, including the thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation. The pulse echoes must be sufficiently short and separated in time for a quantitative analysis [12]. The quantitative methods discussed in this thesis require the time of the echoes from the top of the sample, t_1 , the substrate- sample interface, t_2 , and a reference measurement from the substrate, t_0 , measured beside the sample. A typical ultrasound backscatter signal measured from a sample

at 375 MHz is shown in Figure 2-2.

Figure 2-2: A typical RF signal and the Hilbert-transform envelope from a glass substrate (A) and a cell on a glass substrate (B) at 375 MHz. The pulse echoes from the cell and cell-substrate interface are clearly separated in time.

If the sound velocity in the coupling fluid c_0 is known, the thickness d of the sample can be calculated using

$$d = \frac{c_0}{2} (t_0 - t_1), \quad 2-25$$

and the sound velocity in the sample obtained from

$$c = c_0 \frac{t_0 - t_1}{t_2 - t_1}. \quad 2-26$$

The signal as a function of z -position must then be measured over the region of interest to find the maximum amplitude of the ultrasound signal from the three interfaces; this technique is called a $V(z)$ curve [85]. The transducer is positioned so the focus is below the sample, and the signal is recorded as the transducer is moved up through the sample. The signal from an

interface will be maximized when the ultrasound focus is at that interface. The signal maxima from a $V(z)$ curve of PVDF are A_1 (top of sample), A_2 (sample-substrate) and A_3 (reference) as shown in Figure 2-3.

Figure 2-3: A $V(z)$ curve for 9 μm thick PVDF measured at 375 MHz. The maximum amplitudes from the top of the PVDF (A_1), PVDF-substrate interface (A_2) and a reference (A_3) are easily obtained.

The incident amplitude A_0 can be determined [86] by using

$$A_0 = A_3 \frac{Z_s + Z_0}{Z_s - Z_0}, \quad 2-27$$

where Z_s and Z_0 are the known acoustic impedances of the substrate and coupling fluid, respectively. Then the acoustic impedance of the sample can be obtained using

$$Z = Z_0 \frac{A_0 + A_1}{A_0 - A_1}. \quad 2-28$$

From the measured acoustic impedance Z and speed of sound c , the sample density can then found using

$$\rho = \frac{Z}{c}. \quad 2-29$$

Finally the bulk modulus can be calculated using

$$K = cZ = \rho c^2, \quad 2-30$$

with the assumption that shear waves are negligible in fluids [37]. A quantitative analysis requires the amplitude of both the echoes from the top and bottom of the sample to be resolved throughout a $V(z)$ scan, which is not always possible due to signal attenuation through the sample.

2.1.5 Attenuation

For fluids that have viscous properties, the ultrasound wave will attenuate during propagation. The signal attenuation is due to scattering and absorption of the ultrasound wave as it travels through a medium. Water is typically used as a coupling fluid because it is weakly attenuating, however at the frequencies used in acoustic microscopy, attenuation of the coupling fluid must be taken into consideration [12]. The attenuation can be accounted for by adding a term

$$d\nabla^2 \frac{\partial u}{\partial t}$$

in the wave equation so it becomes

$$\nabla^2 u - \frac{1}{c^2} \frac{\partial^2 u}{\partial t^2} + d \nabla^2 \frac{\partial u}{\partial t} = 0, \quad 2-31$$

where d relates the rate of compression to the viscous relaxation [12]. The equation of a damped harmonic wave

$$u = u_0 e^{-\alpha x} e^{i(\omega t - kx)}, \quad 2-32$$

where α is the attenuation, is a solution to the modified wave equation. The attenuation is directly related to the frequency of the propagating wave according to the relation

$$\alpha = \alpha_0 f^n, \quad 2-33$$

where α_0 is the attenuation coefficient and n is a positive number [76]. For bulk tissue, n is typically between 1 and 2 [87]. The attenuation within the cell can be calculated if the cell-substrate echo A_2 can be measured and the attenuation α_c in the coupling fluid through a distance $2d$ is known [12]. The attenuation within the cell is

$$\alpha = \alpha_c + \frac{1}{2d} \ln \left[\frac{A_0}{A_2} \frac{Z_s - Z}{Z_s + Z} \frac{4ZZ_0}{(Z + Z_0)^2} \frac{Z_s + Z_0}{Z_s - Z_0} \right]. \quad 2-34$$

Waves experience a change in the phase velocity as a function of frequency in most materials, which is called dispersion. Dispersion is due to absorption of the wave energy in the propagating medium; as the wave attenuates and loses energy, the phase velocity decreases [76]. The original Kramers-Kronig (KK) equation relates the phase velocity to the attenuation for electromagnetic waves. This relation was developed into a form for

ultrasound waves

$$\frac{1}{c(\omega)} = \frac{1}{c_0} + \alpha_0' \tan\left(\frac{n\pi}{2}\right) \left(|\omega|^{n-1} - |\omega_0|^{n-1} \right), \quad n \neq 2 \quad 2-35$$

$$\frac{1}{c(\omega)} = \frac{1}{c_0} - \frac{2}{\pi} \alpha_0' \ln\left(\frac{\omega}{\omega_0}\right), \quad n = 2 \quad 2-36$$

where c_0 and ω_0 denote the reference sound velocity and frequency, respectively and n ranges from 0 to 3 [88].

Waves propagating in water experience no dispersion; the sound velocity does not change with frequency [89]. See Appendix A for details on why dispersion is negligible at the frequencies used in acoustic microscopy. However there is a strong quadratic dependence of attenuation with frequency. In equation 2-36, $n = 2$ for longitudinal waves propagating in water. The attenuation also depends strongly on the temperature of the medium. The attenuation in units of dB/cm/MHz as a function of temperature in water for transducers ranging in frequencies from 200 to 1200 MHz are shown in Figure 2-4 [90]. At room temperature, the attenuation at 200 MHz is 88 dB/cm, however, at 1200 MHz the attenuation is 3165 dB/cm. The attenuation decreases with temperature, and the attenuation at 1200 MHz is only 2220 dB/cm at 37°C. The focal length of transducers used for acoustic microscopy are typically 50-500 μm , with the shorter focal lengths used for the GHz lenses. Therefore the expected attenuation for a typical GHz transducer should be 20-30 dB in water, depending on the temperature. While the attenuation is large, modern noise reduction methods and amplification techniques have increased the SNR to enable ultra-high frequency acoustic

microscopy imaging methods.

Figure 2-4: The ultrasound attenuation in water for frequencies ranging from 200 – 1200 MHz.

2.1.6 Rayleigh Waves

Rayleigh waves, or surface acoustic waves, are a superposition of shear and longitudinal waves that travel along a solid surface with the same phase velocity c . Rayleigh waves are slower than both a longitudinal or shear wave [12]. They re-emit energy back to the transducer as they propagate along the surface [91]. If the angle of incidence and emission are equal, the emitted wave will be focused by the lens back towards the transducer and contribute to the signal. Rayleigh waves can increase the contrast of acoustic imaging due to constructive and destructive interference with reflected waves from the sample, but can also create difficulties during a quantitative analysis [12].

Figure 2-5: Propagation of an ultrasound wave from a defocused transducer, creating a Rayleigh wave on the sample surface.

Figure 2-5 shows a transducer with negative defocus (the ultrasound focus is below the sample) generating a Rayleigh wave traveling to the right which emits energy back to the transducer. A single ultrasound wave A travelling down the transducer is focused to focal spot B, below the sample. The Rayleigh wave C will propagate along the sample surface, emitting waves D, E and F back to the transducer. Not all waves will reach the piezo and be converted into a signal, only wave E is focused directly back to the piezo and will contribute towards the signal.

If the signal is measured continuously as a function of transducer position, perpendicular to the sample ($V(z)$ curve), the signal will be a maximum when the focus is at the sample-fluid interface. Oscillations in the recorded signal are observed, which are due to interference of waves emitted from the Rayleigh waves and reflected waves from the surface.

An example $V(z)$ curve from glass at 375 MHz and 1.2 GHz is shown in Figure 2-6.

Figure 2-6: Normalized $V(z)$ curves from glass using a 375 MHz (A) and 1.2 GHz (B) transducer. Oscillations due to Rayleigh wave interference are clearly visible at 1.2 GHz, creating peaks outside the transducer focus.

The oscillation period from the Rayleigh wave contribution as shown in Figure 2-6 can be used to calculate the critical angle at which Rayleigh waves will be generated,

$$z = \frac{\lambda_0}{2(1 - \cos \theta_R)}, \quad 2-37$$

where θ_R is the angle between the wave and the sample and λ_0 is the ultrasound wavelength in the coupling fluid [12]. The velocity of the Rayleigh wave can then be calculated using Snell's law

$$\sin \theta_R = \frac{c_0}{c_R}, \quad 2-38$$

where the subscripts 0 and R denote the sound velocity in coupling fluid and the Rayleigh wave.

The generation of Rayleigh waves depend strongly on the aperture angle of the transducer and the elastic properties of the sample [81]. Transducers with narrow apertures will be less likely to generate Rayleigh waves, and low viscosity materials may be unable to produce measurable Rayleigh waves due to attenuation in the material. Rayleigh waves are generally more observable at 1.2 GHz than at 375 MHz, as shown in Figure 2-6. This is likely due to the larger aperture angle at 1.2 GHz (100° vs. 60°) and the relatively larger signal contribution from the Rayleigh wave compared to the reflected wave from the surface.

2.2 Transducers

It is important to know specific properties of a transducer and how the ultrasound will propagate when selecting a transducer for a particular study. The bandwidth, focal length, aperture angle, center frequency, in addition to internal construction are key parameters in determining the resolution, depth of field, and depth of penetration. Figure 2-7 shows a typical transducer and ultrasound beam with the ultrasound parameters.

Figure 2-7: A transducer showing a typical beam profile and various parameters required for resolution calculations. FL is the focal length and DOF is the depth of field.

The axial resolution of the transducer operated in pulse mode depends on the speed of sound c and bandwidth BW of the pulse, and can be calculated using [76]

$$R_A = \frac{c}{2BW}. \quad 2-39$$

A wide bandwidth is ideal for improving the axial resolution but requires very short pulses which are difficult to create. The lateral resolution is limited by the Rayleigh criterion, which describes the minimum distance two objects must be spaced to be individually resolved. For standard light microscopes, the Rayleigh criterion is

$$R_L = 0.61\lambda \frac{(FL)}{a}. \quad 2-40$$

where FL is the focal length and a is the aperture width [92]. However this criterion is an approximation. For acoustic microscopes, the Rayleigh criterion has a slightly better resolution limit, as the acoustic lens acts as both a transmitter and receiver in contrast to

optical microscopes [93]. Therefore the resolving limitation for acoustic microscopes can be written as

$$R_L = 0.51\lambda \frac{(FL)}{a}. \quad 2-41$$

For a lens with a similar focal length and aperture width (f-number=1), the theoretical resolving limit of optical microscopes is around $0.25 \mu\text{m}$ [94]. The maximum usable frequency in acoustic microscopy under typical use is around 2 GHz due to strong signal attenuation of the coupling fluid. A theoretical resolution limit of about $0.40 \mu\text{m}$ is possible at 2 GHz, however, frequencies around 1 GHz are typically used which has a resolution limit of $0.75 \mu\text{m}$.

Cryogenic acoustic microscopy enables even better resolution due to the low attenuation of ultrasound. Super fluid helium has extremely low attenuation, and a resolution of 15 nm was experimentally obtained using super fluid helium at a frequency of 15.3 GHz [95]. However in practice cryogenic microscopy is not feasible for biological examination due to difficulties in operation and damage to biological samples.

The depth of field describes the regions within a sample which can be focused and resolved. The -3 dB depth of field is given by [93]

$$DOF = 7.2\lambda \left(\frac{FL}{a} \right)^2. \quad 2-42$$

2.3 Assumptions and Approximations

The derivation of the elastic moduli are made on the assumption that the object is

isotropic and homogeneous. This is true of many materials, however, cells are neither isotropic or homogeneous. Cells are complex organisms that are made up of irregularly spaced organelles and proteins with sizes ranging from nanometers to microns [42]. However an assumption was made that the cells are indeed isotropic and relatively homogeneous on the length scale that the measurements are made in this study, up to 4 μm at 375 MHz [96].

Acoustic microscopy uses highly focused transducers, and the assumptions in the time resolved calculations assume plane wave propagation. The waves can be approximated by plane waves at the focal region.

Another assumption is the cells are primarily fluid, and therefore do not support shear waves. The penetration depth a of shear waves in Newtonian fluids is

$$a = \sqrt{\frac{\eta}{\pi \rho f}}, \quad 2-43$$

where η is the viscosity [97]. A quick calculation shows that at 1 MHz, the shear wave penetration depth is on the order of microns. At 1 GHz, the penetration depth is magnitudes less and therefore negligible, and we can neglect shear waves at acoustic microscopy frequencies.

Ultrasound can influence the viscous properties of materials. The main component in cells is water, however some solid and visco-elastic components do exist, such as the proteins and some organelles. Due to the very high frequencies used in acoustic microscopy, the ultrasound waves will have a negligible influence on the viscosity of the cytoplasm elements [37]. Therefore the ultrasound interactions will depend on the elastic parameters and

structural properties of the cells [37].

The intensities used for acoustic microscopy are low enough that there is minimal disruption to the cell and the surroundings [12, 98]. Simulations using similar parameters as our microscope indicate minimal temperature increases at the focus [99]. In addition, the pulsed mode and rapid movement of the transducer during scanning ensures the ultrasound is never focused on one region of a cell for any length of time.

Chapter 3 Methods

3.1 SASAM Optical-Acoustic Microscope

The acoustic microscope used in this study was designed and built by Kibero GmbH (Saarbrücken, Germany). The system is composed of many components, including the optical and acoustic modules, ultrasound electronics, climate controlled systems, and a computer with software for controlling the microscope and a separate computer for running other software packages. A picture of the entire microscope system is Figure 3-1, and each component will be described below.

The microscope is a commercially available IX81 optical microscope manufactured by Olympus which has been modified to allow for acoustic measurements. A rotating column above the sample holder contains an optical condenser for transmission optical microscopy and the acoustic module. During acoustic measurements, the acoustic tip is rotated into position, and the optical microscope operates in reflection mode as shown in Figure 3-2. The reflection optical mode allows for precise alignment of the acoustic tip to specific regions of interest within the sample. The contrast using reflection optical microscopy is poor, therefore the option exists to take high contrast optical images with phase contrast before or after

acoustic measurements by rotating the optical condenser in place.

Figure 3-1: The SASAM acoustic microscope. Climate controlled box (A), optical microscope controls (B), dual monitor computer system (C), climate controller and computer (D), electronics modules and computer (E).

Figure 3-2: The acoustic tip positioned over the sample, ready to be lowered. The transducer (A), sample holder (B), thermocouple (C).

Two different sample holders are used for measurements, which are shown in Figure 3-3. One sample holder is designed to hold standard microscope slides, while the other holds Lab-Tek II chamber slides (NUNC, Germany), which has raised edges to hold biological specimens and liquid. The biological sample holder also has an enclosure with an opening to provide CO₂ gas to maintain cell integrity and to prevent the loss of fluids due to evaporation.

Figure 3-3: The sample holders used in the acoustic microscope. Sample holder that uses Lab-Tek II chamber slides for biological observation (A), sample holder for mounting glass slides (B).

3.1.1 Ultrasound Electronics

Three separate electronics modules are used in the acoustic microscope. The first is the Corvus stage controller for moving the sample under the microscope (ITK Dr. Kassen GmbH, Lahnau, Germany). A joystick is used to move the sample in the x - y plane with a minimum step size of 1.5 nm, allowing for precise alignment of the sample under the transducer.

The second module is a piezo controller ENT400 (Piezosystem Jena GmbH, Jena, Germany), which controls the x , y and z movement of the acoustic tip during scans, and also z -movement of the acoustic tip towards the sample. The tip can be scanned over the sample in step sizes ranging from $0.1\text{ }\mu\text{m}$ to $2\text{ }\mu\text{m}$ in the x and y direction, to a maximum area of $100\times 100\text{ }\mu\text{m}$. The piezo stage is capable of moving the tip within a precision of $0.05\text{ }\mu\text{m}$ in the x , y and z direction, with a range of 25 mm .

The third module is used to control the ultrasound pulses. The module contains two monocyte pulse generators, one at 300 MHz with a 100% bandwidth and the other at 1 GHz with a 100% bandwidth with a 10 Vpp amplitude. The pulse repetition rate is selectable but generally kept at 500 KHz to decrease the time of the measurements. The pulse generator used depends on the frequency of the transducer installed. The RF signal is recorded into memory during a scan using an Acqiris DC252 analog-digital converter capable of digitizing at 8 GHz (Agilent, California, USA), then saved to a file once the scan is complete.

3.1.2 Software

The acoustic microscope uses a software package called Acoustic Investigator, provided by the manufacturer Kibero, to control the scan parameters and data acquisition. The scan parameters such as the step size, signal averaging, scan area, time scale, and measurement method can be selected. The software displays the A-scan in real time during measurements, and the B-scan and C-scan can also be displayed as a function of transducer position. Figure 3-4 shows a screen capture of the Acoustic Investigator software package during a measurement.

Figure 3-4: The Acoustic Investigator program used for data acquisition, showing the A-scan (A) B-scan (B) and C-scan images (C), transducer movement controls (D) and acquisition settings (E).

Another software package provided by Kibero called Acoustic Researcher allows a quick analysis of the saved data. The A-scan, B-scan and C-scan can be displayed, the signal filtered and gated to specific regions of the data and the data can be exported to Matlab for further analysis. Figure 3-5 shows a screenshot of the Acoustic Researcher program.

In addition to the software packages provided by Kibero, numerous scripts written in Matlab were used for further analysis. Scripts were used to subtract the reference signal, display $V(z)$ curves, create C-scan images and other manipulations.

Figure 3-5: A screenshot showing data acquisition using the Acoustic Investigator software package. In this screenshot, five cells were imaged as shown in the C-scan (A). The B-scan was gated to include only the backscatter from the cellular region (B), and the cross-hairs on the C-scan indicate where the A-scan signal is measured from (C).

3.1.3 Image Generation

There are several types of images that can be created using ultrasound. The B-scan image (brightness) is an image most familiar to those in clinical ultrasound. It is a cross-sectional view through the sample, a slice in the x - z plane. It provides details through the depth of the sample.

The c-scan (constant distance) is an overhead view of the sample, measured at a constant focus position. It is a transverse slice through the x - y plane. Due to the penetrating properties of ultrasound, C-scan images at different depths can be created. There are two methods to creating a C-scan image, one is by recording the signal from the substrate, which will illustrate the attenuation of samples placed on the substrate. The other is from the backscatter from the cell. The attenuation C-scan image provides an attenuation map of the sample, or where the strongest attenuation is occurring. The backscatter image displays where echoes are occurring within the sample, or a map of the scattering within the sample.

3.1.4 Temperature Controller

The optical and acoustic components of the microscope system are enclosed in a climate controlled box to maintain stable temperatures during data acquisition, as shown in Figure 3-1. Fluctuations in the temperature can affect the accuracy of the measurements as described in Appendix B. Four openings in the box allow for sample loading and operation of the optical microscope. The optical microscope can optionally be controlled via software on the computer to eliminate the need of opening the doors.

The climate box is interfaced to an external electronics module box made by Kibero GmbH, which is then connected to a computer. The electronics box is connected to ceramic heaters located on three sides and the bottom of the box, and a thermocouple located on the sample holder. Software on the computer uses a feedback loop to set the temperatures of the heaters based on the desired temperature and current temperature inside the box. The software also displays the current temperature, the temperature of each ceramic heater, the

temperature set point and other information related to the algorithm used to set the temperature.

Tests have shown the climate system is able to keep a stable temperature to within 0.02°C over a extended period of time. When operating at GHz frequencies, it is important to keep the temperature as constant as possible because changes in the sound velocity will affect the focus position of the transducer. See Appendix B for details on how the temperature fluctuations can affect the measured signal.

3.2 Transducers

A typical transducer is shown in Figure 3-6. A transducer is usually coupled to sapphire due to the high sound velocity (11500 m/s), low attenuation and robustness. However the transmission power from sapphire to water is very low, due to the differences in the acoustic impedances (the acoustic impedance for sapphire is 44 MRayls, compared to 1.5 MRayls for water). A glass matching layer $\frac{1}{4}$ wavelength thick is typically used to increase the power transmission [12].

The focusing cavity is spherical in shape, with the aperture width and focal length chosen for optimum focusing. The buffer rod is typically made of sapphire not only due to its strength and robustness, but also due to the high index of refraction between the sapphire and water. The buffer rod reduces multiple reflections in the transducer and ensures the signal from the sample does not overlap the internal echoes [12].

Figure 3-6: The three transducers used in this research. From left to right, 200 MHz, 375 MHz and 1.2 GHz.

Center Frequency (MHz)	-6 dB Bandwidth	Focal Length (μm)	Aperture Width (μm)	Aperture Angle ($^{\circ}$)
205	49%	500	500	60
375	42%	350	350	60
1250	30%	50	50	100

Table 3-1: Properties of the three transducers measured at 36°C.

The acoustic microscope is equipped with three transducers as shown in Figure 3-6, with center frequencies of 200 MHz, 375 MHz and 1.2 GHz. The -6 dB bandwidth, focal length, F-number, aperture width and aperture angle are shown in Table 2-1, based on calculations from section 2.2. The pulse and power spectra from these transducers are shown in Figure 3-7.

Figure 3-7: Pulse signal and power spectra for the 200 MHz, 375 MHz and 1.2 GHz transducers as measured from a glass substrate at 36°C.

The 200 MHz and 375 MHz transducers were manufactured by Kibero, while the 1.2 GHz transducer was manufactured by Kraemer Scientific Instruments GmbH (KSI, Herborn, Germany). The construction of the three transducers is similar, the exception being the piezoelectric material and the electronics construction used for the GHz vs. the MHz transducers.

3.2.1 Noise

The composition of cells is mostly water, and water is used as the coupling fluid which results in a very low reflection coefficient from cells. Therefore the backscattered ultrasound intensity from cells is small and efforts must be made to minimize any system noise.

A band stop filter with a frequency range of 88 to 108 MHz was used to eliminate any FM-radio interference (NSBP-108, Mini Circuits, New York, USA). A low pass filter was used to remove any signals lower than 290 MHz (SHP-300+, Mini Circuits, New York, USA). Measurements were averaged 400 times at 375 MHz, and 1000 times at 1.2 GHz to improve the SNR. Signal post-processing was used to remove the reference signal and further reduce noise.

The reference signal is measured adjacent to the cell, however the substrate is typically at a slight inclination. Therefore the tilt must be corrected for accurate quantitative calculations. By measuring the time of the echo from the corners of the substrate, the tilt can be corrected using a system of linear equation described in [39].

Some noise cannot be eliminated from the scans, and careful selection of the

measurement regions must be chosen to avoid overlap of the signal from the sample and system noise. A discussion of other noise and artifacts is located in Appendix C.

3.3 Optical Microscopy

The microscope system is equipped with an advanced optical microscopy system with two light paths. Transmission optical microscopy can be used by rotating the optical condenser over the sample. This allows the option of using optical phase contrast, however, acoustic images cannot be used during transmission images as the acoustic tip is on the same rotating column. Reflection optical and fluorescence microscopy can be used by using a rear light source, with the light reflected from the sample or generated within the sample. It can be used while acoustic scans are in progress. This mode is also used to align the transducer to regions of interest in the sample.

The optical microscope has two imaging paths and several imaging modes. Figure 3-8 shows the microscope and the two light paths. Light path #1 uses transmission light through the sample for viewing. It is used for sample imaging before and/or after acoustic imaging. It cannot be used during acoustic measurements as either the optical condenser or acoustic tip can be above the sample at any one time. Light path #2 uses reflected light from the sample and allows for sample imaging during acoustic scans. Fluorescence imaging can also be used with light path #2.

A monochrome CCD camera (Lumenera, Ottawa, Ontario, Canada) is attached to enable image capture and preview in real time at a maximum resolution of 1392x1040 pixels, and a maximum frame rate of 15 frames per second (fps).

The microscope has three optical objectives in the UIS2 series from Olympus, 10x (UPLanFLN), 20x and 40x (LUCPLanFLN). Three fluorescence filters are also available, UV excitation (model U-MNU2), blue excitation (U-MNB2) and green excitation (U-MWG2).

The contrast of optical and acoustic imaging are very different. Contrast of optical imaging depends on the absorbance and reflectance of light with a sample. Cells are nearly transparent and have poor inherent contrast with standard light microscopy [100]. Acoustic imaging relies on the scattering of sound waves from the sample or their attenuation as they propagate through the sample, which depends strongly on the mechanical properties of the cell and the ultrasound wavelength used.

Light path #1 is desirable for high contrast resolution optical imaging. It can also use a method called phase contrast, which enhances the contrast due to fluctuations in the phase of the light as it travels through the sample. Light path #2 has comparatively poor contrast, however, it allows for simultaneous measurements during acoustic scans.

Ideally the region of interest should be imaged with phase contrast prior to acoustic imaging. Reflection optical imaging can then be used to precisely align the transducer and ensure measurements are recorded in the desired areas.

Figure 3-8: The Olympus IX81 light microscope showing two optical paths, from the light source on the top of the microscope through the sample (A) and from the back of the microscope, reflected from the sample (B) (Image from Olympus).

3.4 Instrument Calibration

The first step in this study was to calibrate and verify the quantitative methods with a known material before measuring specimens with unknown properties, such as cells. This section outlines the methodology verification using polyvinylidene fluoride.

3.4.1 Polyvinylidene fluoride

Polyvinylidene fluoride (PVDF) is a piezoelectric thermoplastic commonly used in transducer fabrication, first used in 1969 [101]. It has been studied extensively over the past thirty years, the mechanical properties of PVDF are well known. PVDF can be manufactured

to very thin thicknesses ($< 9\mu\text{m}$), which is ideal for ultra-high frequency measurements as it is sufficiently thin that the ultrasound won't be attenuated through the sample and is similar to the thickness of cells. Therefore PVDF a well suited material to test and validate the time resolved acoustic microscopy methodology.

Figure 3-9 shows a schematic of the PVDF measurement setup. The PVDF is attached to a glass microscope slide, and the coupling fluid is deposited on the top. Adherent cell lines naturally adhere to the substrate they are grown on, however PVDF does not have any inherent adherent properties. It is important to minimize the gap between the PVDF material and the substrate, as this would introduce errors in the measurements due to the change in the amplitude and time of the ultrasound echoes from the substrate-PVDF interface. Additionally, the presence of air at the interface would introduce errors in the signal amplitude due to the strong scatter and attenuation of air pockets.

Figure 3-9: An illustration of the calibration method. The PVDF was pressed onto the microscope slide, then the coupling fluid was deposited onto the PVDF.

Difficulties were encountered with adhering the PVDF to the substrate. It was found the coupling fluid would seep under the PVDF, causing it to float. Adding weights or taping

the PVDF had little effect, and using glue would add a non-negligible thickness and skew the results. It was found simply pressing the PVDF material onto the glass substrate was sufficient for adherence. The PVDF adhered to the glass like cling-wrap. Optical observations showed the PVDF generally adhered flat to the substrate except in regions where dust, debris or air was trapped between the PDVF and glass. Interference fringes from light between the substrate and PVDF were observed around regions of poor adherence as demonstrated in Figure 3-10. Similar to interference observed with Newton's rings, the spacing of the fringes can be used to determine the gap thickness [102]. Large spacing between the fringes indicate a flat region, or where there is good adherence and minimal gap between the PVDF and substrate. Therefore measurements were chosen on areas where the spacing between the interference fringes was greatest.

A piece of PVDF 3x3cm was used for these calibration studies. The PVDF material used had a large surface area in comparison to the size of the transducer, and it was observed that the center of the PVDF adhered optimally to the substrate, while the edges tended to curl away from the substrate. Therefore measurements were done on the center of the PVDF. A reference signal must be measured from the substrate for a quantitative analysis, however the transducer would have to be moved far from the PVDF measurement to measure the substrate. Inclination of the sample would introduce large errors in the measurement. To reduce errors, the reference measurement was simply measured by removing the PVDF from under the transducer and measuring the reference signal. This ensured the reference and PVDF measurements were made at the same height and position. Since the transducer was not moved during these measurements, the inclination of the sample could be neglected.

Figure 3-10: An optical view of PVDF pressed onto a glass substrate. Interference fringes were used to determine optimum positions for measurements. Position A indicates a small region raised above the substrate. Positions B and C were used for acoustic measurements.

A quantitative analysis was done on a 9 μm piece of PVDF (Measurement Specialties, Hampton, VA, USA) using a 375 MHz transducer. $V(z)$ curves were done through the sample to determine optimum focus locations. The measurement was started with the transducer focus within the substrate, then moved up (positive z -direction) through the PVDF. The signal was recorded in 0.25 μm steps in the z -direction. The PVDF was removed, and the $V(z)$ measurement repeated with the same measured parameters.

3.5 Biological Methods

MCF-7 breast cancer cells obtained from ATCC were cultured for this experiment. Cells were grown in 175 cm² cell culture flasks (Sarstedt, Newton, NC, USA) using Dulbecco's modified eagle's medium (DMEM) cell culture medium (ATCC, Manassas, VA) with 10% fetal bovine serum (FBS) and 0.1% insulin. Cells were incubated at 37°C with 5% CO₂ and were passed every 3 days to maintain exponential growth.

72 hours prior to experimentation, cells were dissociated using trypsin and transferred to Lab-Tek II chambers made of borosilicate glass (Nunc, Germany). After 48 hours of incubation to ensure proper adherence of the cells to the substrate, the medium was replaced with a solution consisting of the DMEM cell culture medium (without FBS or insulin), 3 mg/mL of caffeine (Sigma-Aldrich, Canada) and 20 ng/mL of Paclitaxel (Bristol-Myers Squibb, Montreal, Canada) to induce apoptosis [103-105], then the cells were incubated overnight (approximately 15 hours) prior to experimentation at 37°C with 5% CO₂. During experimentation, cells were kept at a constant temperature of 36°C with 5% CO₂.

A common method to determine the nuclear region of a cell is to stain them with Hoechst 33342, a fluorescent dye which binds to DNA. When excited with ultraviolet light, the dye emits a blue light easily observable with fluorescence optical microscopy. Cells were stained to correlate the area of ultrasound backscatter to regions within the cell.

Chapter 4 Results

4.1 PVDF Calibration Results

Before the quantitative methodology outlined in this thesis can be used on cells, it must first be verified using a known material, PVDF. A typical $V(z)$ curve is shown in Figure 4-1 with the mechanical property measurements. The substrate reference maximum appears to be between the PVDF surface and glass-PVDF interface maxima. This is due to a change in the index of refraction through the PVDF material, as the speed of sound and density are different than the coupling fluid. This results in a tighter beam focus, which requires moving the transducer closer to the sample to reach the substrate. Therefore the substrate reference measurement appears to be higher (measured sooner) than the PVDF-substrate interface.

Five measurements were done using the same method, with the PVDF and transducer repositioned for each measurement. The time of the echo at each z -position was used to calculate the thickness and speed of sound using equations 2-25 and 2-26. The acoustic impedance was then calculated by determining the maximum amplitude from the PVDF surface and substrate reference. Equations 2-29 and 2-30 were then used to calculate the density and bulk modulus. The attenuation was calculated using equation 2-34, and the attenuation in units dB/cm/MHz was calculated using equation 2-33, assuming a linear

Figure 4-1: A $V(z)$ curve of PVDF with the mechanical properties of PVDF are included, showing the % error of the calculated values for the thickness, sound velocity, density and bulk modulus as compared to the values provided by the manufacturer. The top of the PVDF is to the left, the bottom of the PVDF to the right.

frequency dependence ($n=1$). The error was calculated between the values measured and those provided by the manufacturer [106] at each z -position. Figure 4-1 shows the percent error as a function of z -position, throughout the entire $V(z)$ curve. This figure was used to determine the optimum focus position of the transducer for measurements. The speed of sound, density and bulk modulus had the smallest error in the region $z = -2.5$ to $2.5 \mu\text{m}$, where the focus is at the middle of the PVDF. However the thickness had the smallest error in the region $z = -10$ to $-5 \mu\text{m}$, a slightly higher focus position than the other parameters. For

PVDF, the values for the speed of sound, density and bulk modulus were averaged over the region $z = -2.5$ to $2.5 \mu\text{m}$, while the thickness was averaged over -10 to $-5 \mu\text{m}$. The average and standard deviation over the five measurements was calculated for each mechanical property and the values given by the manufacturer are shown in Table 4-1. Agreement of the measured values to the given values were within 4.5%, within the standard deviation. Note the tolerances on the PVDF properties were not given.

Property	Given Value[106]	Calculated Value
Thickness (μm)	9	8.6 ± 0.4
Sound Velocity (m/s)	2200	2211 ± 59
Acoustic Impedance (MRayls)	3.92	3.95 ± 0.09
Density (kg/m^3)	1780	1786 ± 80
Bulk Modulus (GPa)	8.5	8.7 ± 0.2
Attenuation (dB/cm)	-	2571 ± 705
Attenuation (dB/cm/MHz)	-	6.9 ± 1.9

Table 4-1: A comparison of the calculated mechanical properties of PVDF versus those provided by the manufacturer.

PVDF is a solid, therefore shear waves and Rayleigh waves could be produced in the sample which could affect the amplitude of the measured signal. It is unknown what effect mode conversion has with the PVDF measurements. However a comparison of the measured values to accepted values showed good agreement, and it is unlikely that mode conversion has a strong effect on these measurements at 375 MHz.

4.2 Cellular Measurements

Acoustic microscopy was used to measure the RF signal from MCF-7 cancer cells at

200 MHz, 375 MHz and 1.2 GHz using the same methods as outlined during the PVDF verification. The transducer was positioned over cells using optical microscopy as shown in Figure 4-2. The ultrasound backscatter from the cells and substrate were measured as a function of time, and $V(z)$ curves were performed over areas of strong backscatter within the cell.

Figure 4-2: The 375 MHz transducer positioned over cells for measurements. The cells to be measured (A) are under the spherical cavity (B) of the transducer (C).

For a quantitative analysis, a backscatter signal must be measurable from the region of interest. The amplitude must be sufficiently strong to accurately determine the time of the echo reflection. In addition, the echo from the cell and the substrate must be separated in time so there is no overlap. An example of a suitable backscatter signal from the center of a cell is

shown in Figure 4-3. The backscatter amplitude from cells was typically 10 – 25 mV.

Figure 4-3: The ultrasound signal as a function of time from the center of a MCF-7 cell. The ultrasound echo from the cell is clearly separated in time from the echo from the glass substrate.

4.2.1 Mechanical Properties of Untreated Cells

Using equations 2-25 to 2-30, the maximum amplitude measured from the $V(z)$ curves, and the time of the ultrasound backscatter echoes, the thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation were calculated over areas of strong backscatter for untreated MCF-7 cells.

Table 4-2 summarizes the calculations for four different MCF-7 cells measured at 375 MHz. This is the first time all these properties have been measured together using one method for the same non-fixed cell. The sound velocity measurements are in general agreement with published data using other cell lines, which measured a sound velocity of

1534.5 m/s in Hela cells [39] and 1571 m/s in human aortic heart muscle [35] using acoustic microscopy. A lack of published data on individual cell properties prevent a comparison of the other parameters.

Using equation 2-33, the frequency dependence was removed from the attenuation assuming $n = 1$, a common value used for bulk tissue [87]. Table 4-2 shows the attenuation in units of dB/cm, and also dB/cm/MHz with the frequency dependence removed.

Property	Cell 1	Cell 2	Cell 3	Cell 4
Thickness (μm)	16.1	11.9	12.4	11.5
Sound Velocity (m/s)	1613	1574	1557	1585
Acoustic Impedance (MRayls)	1.60	1.60	1.55	1.58
Density (kg/m^3)	994	1016	994	996
Bulk Modulus (GPa)	2.59	2.52	2.41	2.50
Attenuation (dB/cm)	585	536	671	585
Attenuation (dB/cm/MHz)	1.56	1.43	1.79	1.56

Table 4-2: The mechanical properties of MCF-7 cells measured using time resolved acoustic microscopy.

4.2.2 Mechanical Properties of Treated Cells

Using the method described in the previous section, the thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation were calculated at the center for treated MCF-7 cells before and after apoptosis. Seven treated cells that had not undergone apoptosis were measured (denoted unresponsive, Table 4-3), and five rounded-up cells (denoted responsive, Table 4-4). Between one and three measurements were made at each cell. The average and standard deviation of these values are summarized in Table 4-5.

Property	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7
Thickness (μm)	14.7	16.5	17.9	11.8	8.1	13.6	13.3
Sound Velocity (m/s)	1561	1572	1561	1552	1593	1578	1542
Acoustic Impedance (MRayls)	1.538	1.544	1.550	1.556	1.567	1.548	1.548
Density (kg/m^3)	986	982	993	1003	984	981	1004
Bulk Modulus (GPa)	2.40	2.43	2.42	2.42	2.50	2.44	2.39
Attenuation (dB/cm)	360	356	353	390	476	379	390
Attenuation (dB/cm/MHz)	0.96	0.95	0.94	1.04	1.27	1.01	1.04

Table 4-3: The mechanical properties of treated, but unresponsive MCF-7 cells.

Property	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5
Thickness (μm)	17.1	17.0	18.2	15.6	18.4
Sound Velocity (m/s)	1567	1564	1559	1565	1550
Acoustic Impedance (MRayls)	1.550	1.553	1.529	1.522	1.525
Density (kg/m^3)	989	994	981	972	984
Bulk Modulus (GPa)	2.43	2.43	2.38	2.38	2.36
Attenuation (dB/cm)	488	773	750	698	469
Attenuation (dB/cm/MHz)	1.30	2.06	2.00	1.86	1.25

Table 4-4: The mechanical properties of treated, responsive apoptotic cells.

Property	Unresponsive Cells	Responsive Cells
Thickness (μm)	13.6 ± 3.1	17.3 ± 1.6
Sound Velocity (m/s)	1582 ± 19	1574 ± 19
Acoustic Impedance (MRayls)	1.56 ± 0.01	1.55 ± 0.02
Density (kg/m^3)	987 ± 14	984 ± 20
Bulk Modulus (GPa)	2.47 ± 0.03	2.44 ± 0.03
Attenuation (dB/cm)	405 ± 79	653 ± 135
Attenuation (dB/cm/MHz)	1.08 ± 0.21	1.74 ± 0.36

Table 4-5: Mechanical properties of MCF-7 cells at 375 MHz.

The sound velocity, acoustic impedance, density and bulk modulus were similar within experimental error before and after apoptosis. The thickness of apoptotic cells were higher at $17.3 \pm 1.6 \mu\text{m}$, compared to $13.6 \pm 3.1 \mu\text{m}$ for unresponsive cells. The attenuation calculated for apoptotic cells measured above the nuclear region was $653 \pm 135 \text{ dB/cm}$, compared to $405 \pm 79 \text{ dB/cm}$ for unresponsive cells. Using equation 2-33, the frequency dependence was removed from the attenuation assuming $n = 1$. The attenuation after removing the frequency dependence was $1.74 \pm 0.36 \text{ dB/cm/MHz}$, compared to $1.08 \pm 0.21 \text{ dB/cm/MHz}$ for unresponsive cells. The attenuation increased by 75% after apoptosis, despite the other mechanical properties measured remaining unchanged.

4.2.3 Acoustic and Optical Comparison

Acoustic images of MCF-7 cells were compared to optical images to examine the source of the ultrasound backscatter. Cells were stained with Hoechst 33342, which emits a blue fluorescence when bound to DNA, to delineate the nucleus. Optical fluorescence images and phase contrast images measured before the acoustic scans were compared to the acoustic attenuation and backscatter images measured at 200 and 375 MHz, as shown in Figure 4-4. At 200 MHz, locations of strong backscatter are spatially coincident with locations of nuclear staining, suggesting that the scattering originates from this region. In addition, the signal from the cell and substrate generally overlap, preventing a quantitative assessment at 200 MHz. At 375 MHz, the center of the cell generally remained the dominant scattering source.

Figure 4-4: Optical and acoustic comparison of two MCF-7 cells measured at 200 MHz (left) and 375 MHz (right). Each frame consists of optical phase contrast (A), optical fluorescence (B), acoustic attenuation (C) and acoustic backscatter (D) images.

4.2.4 Acoustic Measurements of Apoptotic Cells

MCF-7 cells were treated with Paclitaxel, a chemotherapeutic agent to induce apoptosis. After 15 hours, these cells were measured using the 375 MHz and 1.2 GHz transducers before and after morphological changes were observed. Optical and ultrasound attenuation and backscatter images of typical cells in both stages measured at 375 MHz are shown in Figure 4-5.

Figure 4-5: A comparison of the optical and acoustic images of a treated cell showing membrane blebbing, an early sign of apoptosis (top) and a responsive apoptotic cell (bottom). From left to right: Optical phase contrast image, acoustic backscatter image, acoustic attenuation image. The images shown in this figure are from two different cells, however the scales are similar allowing direct comparison of the acoustical properties measured.

Figure 4-6 shows an enlarged view of the backscatter from the unresponsive and responsive cells depicted in Figure 4-5. In addition to the C-scan backscatter image, a B-scan, a cross sectional profile of the backscatter through the area indicated by the arrows, is shown. An increase in the height of the responsive cell was observed. In addition, regions with large backscatter variations from within the cell was observed.

Figure 4-6: A comparison of the backscatter signal from the unresponsive cell (left) and the responsive cell (right) from Figure 4-5. The c-scan backscatter image is shown on top, and the b-scan showing the scattering regions from the cell is on the bottom. The location of the b-scan is indicated by the arrow.

The backscatter signal amplitude measured from apoptotic cells varied between 10 and 25 mV (Figure 4-7), similar to what was observed with untreated cells (Figure 4-3). While the signal amplitude was similar for cells in both states (10-25 mV), the spatial locations within the cell in which significant backscatter was observed increased for apoptotic cells, which is demonstrated in the backscatter images in Figure 4-5.

Figure 4-7: An RF-signal from a typical apoptotic cell. Scattering from the top of the cell and the substrate are clearly visible.

Apoptotic cells were also imaged using the 1.2 GHz transducer and compared to optical images and acoustic measurements made at 375 MHz. Figure 4-8 shows a comparison of the acoustic attenuation images made using the 375 MHz and 1.2 GHz transducers. The 1.2 GHz transducer showed detail not resolved at lower frequencies. The nucleus and cytoplasm regions were clearly delineated, and the attenuation in regions containing the nucleus appeared to be higher than the cytoplasm. Additionally, protrusions from the nucleus into the cytoplasm, and occasionally outside the cell were observed as shown in Figure 4-9. The backscatter was not reliably detected due to the low SNR, therefore a quantitative analysis was not possible at 1.2 GHz.

Figure 4-8: A comparison of acoustic attenuation images of an apoptotic cell at 375 MHz (A), 1.2 GHz (B) and compared to an optical image (C). The same cell was used for each scan.

Figure 4-9: Attenuation images of two different cells measured at 1.2 GHz. Protrusions from the nuclear region into the cytoplasm, and outside of the cell are clearly visible.

4.2.5 Acoustic Variations Over Time

Extensive activity within the cell was visually observed for a period of time after apoptosis. Figure 4-10 shows acoustic attenuation and backscatter images from an apoptotic cell measured one minute apart with the focus at the substrate, and frames from an optical movie captured at the same time as the acoustic data. The intensity scales are the same for the

each type of ultrasound images for comparison. The acoustic attenuation images appear unchanged throughout the time period, indicating minimal attenuation variations within the timescale of this measurement. The backscatter images however show variations with each measurement. The spatial patterns of backscatter and intensity variations indicate extensive activity occurring within the cell. This correlates to optical movies taken during the acoustic measurements, where membrane blebbing and other morphological changes were visible.

Figure 4-10: Optical and acoustic images measured 1 minute apart. Optical images (top row), acoustic attenuation images (middle row), acoustic backscatter images (bottom row). Acoustic scans were made with a 1 μm step size over an area of 40x40 μm and took 50 seconds to acquire.

Optical movies show morphological variations occur in apoptotic cells over a time period of seconds. The timeframe of acoustic measurements is too slow to show a sequence of activity within the cell at the time scale of seconds. We can only conclude variations in the backscatter were observed over the time period of minutes. Larger step sizes or lower scan

areas could improve the scan time, but at a cost of image resolution.

4.2.6 Temporal Signal Variations

The activity within a cell was measured by fixing the transducer over a single region and recording the signal every 10 seconds. The signals were measured before and after apoptosis for four cells in each state. The integrated backscatter intensity over the cellular region for each RF line were calculated and plotted as a function of time, as shown in Figure 4-11. To determine variations in the signal over time, the correlation coefficient of the RF signal from the cell was calculated for each cell by comparing the first RF signal measured to each subsequent RF signal for that cell. A correlation coefficient of 1 indicates perfect correlation, or no variations in the signal. The correlation coefficient plotted as a function of time is shown in Figure 4-12. A summary of the average correlation coefficient, integrated intensity and maximum amplitude over the time period of the measurements are in Table 4-6, where the standard deviation indicates the variations within the measured values. In addition, the correlation coefficient, integrated intensity and maximum amplitude for each cell are shown in Table 4-7 for untreated cells and Table 4-8 for treated cells undergoing apoptosis.

The average correlation coefficient for unresponsive cells was 0.93 ± 0.05 , compared to 0.68 ± 0.17 after apoptosis. The lower correlation coefficient and higher standard deviation indicate increased variations in the signal after apoptosis. In addition, the average integrated intensity was lower for apoptotic cells, at 0.57 ± 0.14 compared to 0.75 ± 0.10 for unresponsive cells. The maximum amplitude of the signal from the substrate did not show much variation, at 0.529 ± 0.003 V for apoptotic cells, compared to 0.498 ± 0.004 V for cells

that did not respond. This indicates minimal attenuation variations are occurring over time in comparison to the backscatter intensity.

Figure 4-11: The integrated backscatter intensity measured every 10 seconds for 900 seconds. (A) Four untreated cells, (B) four treated cells undergoing apoptosis.

Figure 4-12: The correlation coefficient of the integrated backscatter intensity. (A) Four untreated cells, (B) four treated cells undergoing apoptosis.

Property	Untreated	Treated
Correlation Coefficient	0.93 ± 0.05	0.68 ± 0.17
Normalized Integrated Intensity	0.75 ± 0.10	0.57 ± 0.14
Maximum Amplitude from Substrate	0.529 ± 0.003	0.498 ± 0.004

Table 4-6: Temporal analysis results of MCF-7 cells.

Property	Cell 1	Cell 2	Cell 3	Cell 4
Correlation Coefficient	0.89 ± 0.09	0.98 ± 0.01	0.97 ± 0.02	0.87 ± 0.07
Integrated Intensity	0.66 ± 0.11	0.76 ± 0.14	0.85 ± 0.07	0.72 ± 0.08
Maximum Amplitude	0.651 ± 0.004	0.644 ± 0.004	0.249 ± 0.002	0.571 ± 0.004

Table 4-7: Temporal analysis of untreated MCF-7 cells.

Property	Cell 1	Cell 2	Cell 3	Cell 4
Correlation Coefficient	0.59 ± 0.18	0.61 ± 0.15	0.75 ± 0.15	0.76 ± 0.20
Integrated Intensity	0.70 ± 0.11	0.53 ± 0.14	0.56 ± 0.11	0.49 ± 0.18
Maximum Amplitude	0.533 ± 0.003	0.588 ± 0.003	0.300 ± 0.004	0.570 ± 0.007

Table 4-8: Temporal analysis of treated MCF-7 cells undergoing apoptosis.

Chapter 5

Discussion and Future Work

5.1 Discussion

This thesis presents a newly developed method to measure the mechanical properties of cells such as the sound velocity, acoustic impedance, density, bulk modulus and attenuation using one technique, time resolved acoustic microscopy. While the theory for time resolved methods are well established, technological barriers have prevented utilization of these methods until now. The combination of acoustic and optical components allows for optical verification of the location of the acoustic measurements as well as cellular and sub-cellular feature identification, an advantage over other acoustic microscopes without this capability.

The methodology was first verified using a material with known properties, PVDF. It was found the properties measured at 375 MHz agreed to within 4.5% of the actual properties as provided by the manufacturer.

For the first time, the properties of cells were measured at 375 MHz using one single method. Four cells were measured, and the sound velocity ranged from 1557 to 1613 m/s,

which is similar to the sound velocity of other studies. Human aortic smooth muscle cells were measured at 1571 m/s using an interference fringe method [35], xenopus tadpole hearts were measured at 1.5 to 1.7 m/s using the $V(f)$ method [107], and HeLa cells were found to have a sound velocity of 1535 ± 34 m/s [39] using time resolved acoustic microscopy. While there have been many studies examining the properties of bulk tissue, a lack of published data prevents a comparison of single cell properties where parameters used in the calculations have not been estimated.

The properties of cells undergoing apoptosis were measured at 375 MHz using the time resolved acoustic microscopy technique, which is the first time the properties of cells undergoing this biological process have been measured. It was found the sound velocity, acoustic impedance, density and bulk modulus did not change within experimental error for cells before and after apoptosis. After apoptosis, the thickness increased on average from 13.6 ± 3.1 μm to 17.3 ± 1.6 μm , and the attenuation increased from 1.08 ± 0.21 dB/cm/MHz to 1.74 ± 0.36 dB/cm/MHz. Despite these structural changes, the mechanical properties other than the attenuation did not change during apoptosis for this cell line.

Observations of apoptotic cells using 1.2 GHz showed increased detail over 375 MHz. The backscatter signal was not detectable at 1.2 GHz, and a quantitative assessment of the mechanical properties was not possible. However the attenuation images showed clear delineation of the cell membrane and nuclear region. In addition, protrusions from the nucleus into the cytoplasm, and outside of the cell were observed. This is the first time apoptotic cells have been imaged with high resolution acoustic microscopy showing these details.

A comparison of the optical and acoustic attenuation and backscatter images of apoptotic cells measured one minute apart indicate that variations within the cell are occurring during apoptosis. The attenuation within the cell was not changing after apoptosis occurred, however, the ultrasound backscatter signal showed extensive variations throughout the measurement period. Optical observations of apoptotic cells showed rapid morphological variations of the cell, such as membrane blebbing, on a time scale of seconds. This indicates the cell is undergoing structural changes during apoptosis. To further investigate these variations, temporal variations in the RF-signal from the center of apoptotic cells was measured every 10 seconds for 900 seconds. An increase in the variations of the backscatter signal was observed for apoptotic cells, in contrast to treated cells that were not responsive. The correlation coefficient was used to measure the variations in the signal. Apoptotic cells had an average correlation coefficient of 0.68 ± 0.17 , compared to 0.93 ± 0.05 for non-responding cells. This indicates the non-responding cells have minimal variations throughout the measurement period, in contrast to responding cells, which have extensive variations in the RF-signal.

The measurements of cells before and after apoptosis was observed indicate that in addition to morphological variations in the cell membrane observed visually, acoustic measurements indicate extensive variations also occur within the cell. There appears to be a clear delineation between the cytoplasm and the nucleus, and extensive activity is occurring within the nucleus.

5.2 Conclusions

This thesis presents a method to measure the mechanical properties of MCF-7 cancer cells using time resolved acoustic microscopy, after first verifying the methodology using a material with known properties, PVDF. We have successfully used the time resolved method to measure the mechanical properties of cells before and after apoptosis, in which no difference in the speed of sound, acoustic impedance, density or bulk modulus were observed within experimental error. However an increase in the thickness and attenuation was observed.

Rapid morphological changes were observed in cells undergoing apoptosis, as indicated by variations in the RF-backscattered signal over a period of seconds and minutes. These acoustic measurements correlate to optical observations of the cell. This indicates extensive activity is not only occurring on the cellular surface, but structural changes are occurring within the cell on a time scale of seconds during apoptosis.

The validation of the time resolved methods using PVDF, and measuring the mechanical properties of cells during apoptosis confirms that time resolved acoustic microscopy is capable of examining the cellular microstructure of cells, and in particular, during a time-sensitive biological process such as apoptosis.

5.3 Future Work

Increasing the frequency of the transducer used in the time resolved methods would

decrease the spatial resolution, and allow for a more accurate determination of the mechanical properties of cells. With increased resolution and reduced errors, a difference in the mechanical properties may be observed before and after apoptosis. In addition, as clear delineation of the cytoplasm and nucleus was observed at 1.2 GHz but not at 375 MHz, measurements of the cytoplasm and nucleus with GHz frequencies would enable a quantitative assessment of how these areas change during treatment.

Measurements can be performed on other cell lines during treatment, in particular acute myeloid leukemia (AML) cells, which have been studied extensively at high frequencies (20-60 MHz) previously in this laboratory. The methodology presented in this thesis can also be used for other time sensitive biological processes, such as mitosis, locomotion and exposure of cells to other types of chemotherapeutic agents. The penetrating nature of ultrasound would give insight into the structural changes occurring within the interior of the cell during these processes.

Knowledge of the mechanical properties as a function of the state of the cell will allow modeling and simulations of ultrasound scattering from cells and cell ensembles. These values are required as input to theoretical models of scattering currently being developed in our laboratory [32].

Very little work has been done on scattering from microspheres and microbubbles at the frequencies used in this work. Measuring the response of these materials at ultra-high frequencies using time resolved acoustic microscopy would increase our understanding of ultrasound scattering of micrometer-sized objects. The acoustic measurements can be

combined with optical observations of the microspheres and microbubbles to accurately determine how ultrasound scattering occurs as a function of object size and frequency. These results would aid numerical simulations and comparisons to theoretical models described above.

Appendix A: Dispersion in Water

The speed of sound in water was first measured by Jean-Daniel Colladon and Charles-Francois Sturm on a lake in Switzerland in 1826 [108]. Two boats a specific distance apart were used in the experiment. On one boat, a bell was rung in the water and gunpowder ignited at the same moment. Observers on the second boat recorded the time between seeing the flash from the gunpowder and hearing the bell, and consequently calculated the sound velocity in the water.

Great strides have been made since in measuring the sound velocity in different media. There have been many studies showing the speed of sound is constant from 1-60 MHz and dispersion is negligible within this frequency range [109-111], however, there have not been any studies at GHz frequencies. It is unknown what effect dispersion has at such frequencies and it is important to know due to the high frequencies used in acoustic microscopy.

Davidovich *et al* measured the attenuation slope in water using frequencies from 300 to 3000 MHz [89]. The measured values are constant throughout this range, however there is an error of 5-8% associated with these measurements. Since the attenuation appears constant throughout this range, and attenuation is a requirement for dispersion, it follows that the speed of sound must be constant and dispersion is absent (or negligible) as a function of

frequency. A quick examination into theoretical dispersion can confirm these measurements.

The Kramers-Kronig equations discussed in section 2.1.5 relate the absorption coefficient and phase speed of the ultrasound wave

$$\frac{1}{c(\omega)} = \frac{1}{c_0} + \alpha_0 \tan\left(\frac{n\pi}{2}\right) \left(|\omega|^{n-1} - |\omega_0|^{n-1} \right), \quad n \neq 2, \quad \text{A-1}$$

$$\frac{1}{c(\omega)} = \frac{1}{c_0} - \frac{2}{\pi} \alpha_0 \ln\left(\frac{\omega}{\omega_0}\right), \quad n = 2. \quad \text{A-2}$$

Since water has a value of $n=2$, $\tan(\pi) = 0$ and theoretically dispersion is absent. However the relation may not be exactly 2, so there may be a small dispersion effect that is unaccounted for. This small effect will be approximated using a theoretical estimation. The equation of a plane wave, accounting for viscous loss, can be written as

$$\frac{1}{c_0^2} \frac{\partial^2 \phi}{\partial t^2} - \frac{\partial^2 \phi}{\partial x^2} = \kappa \left(\eta_B + \frac{4}{3} \eta \right) \frac{\partial}{\partial t} \left(\frac{\partial^2 \phi}{\partial x^2} \right), \quad \text{A-3}$$

where c is the sound velocity, x is the position, κ is the compressibility, η is the viscosity and η_B is the bulk viscosity [76]. A solution to this equation, assuming the approximation

$$1 \gg j\omega k \left(\eta_B + \frac{4}{3} \eta \right) \quad \text{A-4}$$

holds is

$$c_0 \approx c_0 \left[1 + \frac{3\omega^2 \kappa^2}{8} \left(\eta_B + \frac{4}{3} \eta \right)^2 \right]. \quad \text{A-5}$$

The adjustment factor

$$\left[1 + \frac{3\omega^2\kappa^2}{8} \left(\eta_B + \frac{4}{3}\eta \right)^2 \right] \quad \text{A-6}$$

is the modification to the speed of sound for the given parameters. We can use this equation to calculate the sound velocity adjustment factor as a function of frequency. At 37°C and a viscosity of 0.000692 kg/m/s, bulk viscosity of 0.001944 kg/m/s, and compressibility of 4.4×10^{-10} Pa was used to determine the adjustment factor for different frequencies. Additionally the assumption in equation A-4 holds true over the frequency range 1 to 1000 MHz.

At 1000 MHz and 37°C, the adjustment factor is 0.002%, resulting in a speed of sound adjustment of only 0.036 m/s. At 100 MHz, the speed of sound adjustment is 0.0004 m/s. The adjustment factor decreases with decreasing frequency, and at 10 MHz and below, the change in the speed of sound is negligible. From experimental work on attenuation, and theoretical calculations, we can conclude that dispersion has a negligible effect at the frequencies used in acoustic microscopy.

Appendix B: Signal Temperature Dependence

The temperature has a direct effect on the sound velocity in water. An equation was developed that describes how the velocity changes as a function of temperature T [112]

$$c = 1402.4 + 5.01T - 0.055T^2 + 0.00022T^3. \quad \text{B-1}$$

From 0 to 60°C, the speed of sound increases with increasing temperature, as shown in Figure B-1. The change in sound velocity will have a direct effect on the focus of the transducer. As the speed of sound changes, the position of the transducer must be moved to maintain the focus on the sample.

The effect of temperature on focus position was examined by measuring $V(z)$ curves from a glass substrate at 1.2 GHz as the temperature drifted slowly over a period of 30 minutes. It was assumed the temperature fluctuations were slow enough that the temperature of the coupling fluid and the air temperature as measured by the thermocouple were the same.

Figure B-5-1: The effect of temperature on sound velocity.

Figure B-2 shows six $V(z)$ curves measured over a temperature range of 0.42°C , from 37.04°C to 37.46°C . The focus shifted by $5.5\text{ }\mu\text{m}$ over this temperature range. At 1.2 GHz , this focus shift is unacceptable as the depth of field is only $12\text{ }\mu\text{m}$. This shift would have detrimental consequences when trying to calculate the mechanical properties of samples, which are dependent on not only the time of the echoes, but the amplitudes. For a focus stability within $0.5\text{ }\mu\text{m}$, a temperature stability of 0.05°C is required. Ideally the better the stability, the more accurate the results.

Figure B-5-2: $V(z)$ curves from glass using a 1.2 GHz transducer at six different temperatures.

The temperature stability at 375 MHz was also investigated, by measuring the time of the echo from a glass substrate as a function of temperature as shown in figure B-3. The measurements were over a temperature shift of approximately 1°C , from 26.6°C to 27.6°C . Over such a short temperature range, the dependence of the sound velocity on temperature can be approximated as a linear dependence.

Figure B-5-3: The time of the ultrasound echo from a glass substrate at 375 MHz as a function of temperature and a line of best fit.

The time of the peak amplitude shifted from 1.514 μs to 1.484 μs over 1°C, a total time difference of 0.030 μs . Additionally, the signal amplitude decreased from 0.6 V to 0.13 V during this drift. Such shifts would introduce errors in the mechanical calculations required for this research.

Appendix C: Noise and Artifacts

Noise and artifacts can be introduced into the system through various means, such as cabling impedance mismatches, RF interference, electrical noise and others [12]. Many can be minimized through careful selection of materials and filters, as discussed in section 3.2. However there is a major source of noise that cannot be eliminated, which is due to the ultrasound propagation through the transducer rim.

Figure C-5-4: (Left) Transducer showing the path length of a wave through the rim (B-C) and through the lens curvature (A-C). (Right) RF-signal showing the rim artifact and substrate signal.

Figure C-1 shows a transducer system measuring a sample with the path length of two waves, one through the rim and one through the lens, and the resulting RF signal. In Figure C-1, the main substrate echo and the rim echo are clearly observed. This second signal is not

from the focused ultrasound through the transducer lens, it is the substrate echo from the ultrasound passing through the rim of the transducer [113].

The artifact appears to be in front of the substrate echo, despite that the path length of the wave is the same through either the rim or the lens. The body of the transducer is made of sapphire, which has a sound velocity of 11150 m/s, compared to the coupling fluid, water, which has a sound velocity of 1500 m/s at room temperature [113]. During the path A-B, the wave travelling through the rim will be in sapphire, while the wave through the lens will be in water. Since the speed of sound is greater in sapphire than water, the echo from the substrate measured through the rim will appear to be ahead of the echo measured through the lens.

This lens artifact echo is always a constant time in front of the main echo. It cannot be removed by a background subtraction. When a cell or object is placed onto the substrate to be measured, the echo must be sufficiently separated from the substrate echo to perform quantitative calculations, however, it must not overlap the rim echo. For cells with a thickness of 10-20 μm , this has not been an issue, however for anything thicker, subtraction of this rim echo must be considered.

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