

A comparison of cellular ultrasonic properties during apoptosis and mitosis using acoustic microscopy

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Abstract—Ultra-high frequency acoustic microscopy methods were used to determine the cell thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation of untreated cells during interphase and metaphase, and cells treated to induce apoptosis. Hoechst 33342, Annexin-V and propidium iodide fluorescent stains were used to identify the cell cycle stage and early/late stage apoptosis. A statistically significant increase in thickness (11.0 to 18.9 μm), decrease in bulk modulus (2.45 to 2.43 GPa) and attenuation (1.44 to 1.10 dB/cm/MHz) was observed between cells in interphase and metaphase. A statistically significant increase in thickness (11.0 to 15.3 μm), sound velocity (1570 to 1588 m/s), bulk modulus (2.45 to 2.47 GPa), attenuation (1.44 to 2.00 dB/cm/MHz) and decrease in density (994 to 979 kg/m^3) was observed between interphase and early apoptotic cells. A statistically significant decrease in thickness (15.3 to 12.5 μm), sound velocity (1588 to 1555 m/s), acoustic impedance (1.554 to 1.537 MRayls), bulk modulus (2.47 to 2.39 GPa), attenuation (2.00 to 1.40 dB/cm/MHz) and increase in density (979 to 988 kg/m^3) was observed between early and late stage apoptotic cells. A total of 110 cells were measured, 34 interphase, 21 metaphase, 29 early apoptotic and 26 late apoptotic. The cellular properties of cells in interphase and metaphase did not significantly change, however there are significant differences between interphase and early apoptosis, and in particular early and late stage apoptosis, indicating considerable structural or organizational rearrangement is occurring.

I. INTRODUCTION

Cells undergo various biological processes such as mitosis and apoptosis during their lifetime. Mitosis is a process of life, where an individual cell creates two identical daughter cells. In contrast, apoptosis is a method of cell death that occurs in a controlled method to minimize disruption to surrounding cells [1].

Deregulation of apoptosis can result in a variety of diseases, including cancer [2]. Many cancerous tumors grow due to a mutation that inhibits apoptosis, and chemotherapeutic treatments are designed to induce apoptotic functionality. Interfering with the mitotic cycle is a common method of inducing apoptosis in cancer cells [3]. Understanding how various cellular properties change during mitosis and apoptosis would aid in monitoring cancer treatments and developing new chemotherapeutic treatments.

Techniques used to determine cellular properties include ultrasound [4], atomic force microscopy, intracellular particle tracking and micropipette aspiration [5]. Ultrasound has an advantage over other techniques, as it can non-invasively probe living cells during various biological processes.

Ultrasound has been used to measure cellular properties on small tissue samples using high frequencies (10–60 MHz) [6]. An increase in ultrasonic backscatter [7] and attenuation [8] was observed for apoptotic cells compared to untreated cells. However it is difficult to accurately study apoptosis on these length scales, as it would require all cells within the region to be in a specific cell state (apoptotic or the same mitosis phase). In tissue, they may contain other types of cells (such as blood vessels). Individual cells cannot be probed at these frequencies, and mitosis would be impossible to study using these methods.

Ultra-high frequency ultrasound (100+ MHz) has been used to study tissue [9] and individual cells [10]. However these studies are usually limited to measurements of cell thickness, sound velocity and attenuation. Previous research by the authors combined time resolved and $V(z)$ ultrasound methods to determine the thickness, sound velocity, acoustic impedance, density, bulk modulus and acoustic attenuation of individual living cells using a single measurement technique [11]. A statistically significant increase in thickness and attenuation was observed before and after apoptosis, however a relatively small sample size was used (12 cells). The goal of this research is to enhance the previous study by using a larger sample size and compare two different biological processes, mitosis and apoptosis. The aim of this study is to determine how these properties change between interphase (the normal cellular state), metaphase (mitosis phase), and early/late stage apoptosis of cancer cells.

II. THEORY

Acoustic microscopy can be used to image and quantitatively determine the ultrasonic properties of individual cells using a combination of time resolved and $V(z)$ methods. The transducer is positioned directly above the cell to be measured, as shown in fig. 1. The time of the echoes from the cell, t_1 , cell-substrate interface, t_2 , and a reference measurement

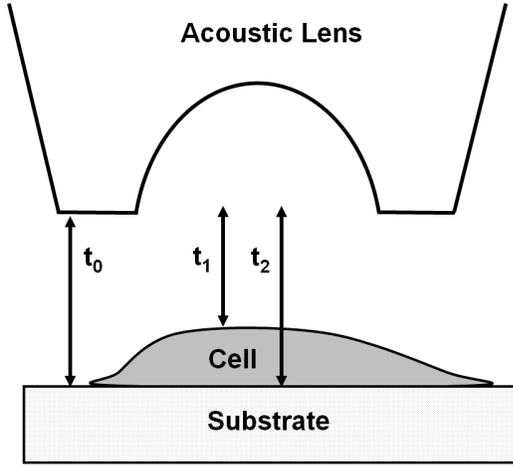


Figure 1. The system schematic. t_1 and t_2 are recorded with the transducer positioned above the cell. The transducer is moved beside the cell to measure the reference t_0 from the substrate.

from the substrate, t_0 , as well as the signal amplitudes from each interface (A_1 , A_2 and A_0 , respectively) can be used to calculate the thickness t , sound velocity c , acoustic impedance Z , density ρ , bulk modulus K and attenuation α using the following equations

$$d = \frac{c_0}{2}(t_0 - t_1), \quad (1)$$

$$c = c_0 \frac{t_0 - t_1}{t_2 - t_1}, \quad (2)$$

$$Z = Z_0 \frac{A_0 + A_1}{A_0 - A_1}, \quad (3)$$

$$\rho = \frac{Z}{c}, \quad (4)$$

$$K = cZ = \rho c^2, \quad (5)$$

$$\alpha = \alpha_c + \frac{1}{2d} \ln \left[\frac{A_0}{A_2} \frac{Z_s - Z}{Z_s + Z} \frac{4ZZ_0}{(Z + Z_0)^2} \frac{Z_s + Z_0}{Z_s - Z_0} \right], \quad (6)$$

where c_0 is the sound velocity in the coupling fluid, Z_s is the acoustic impedance of the substrate, Z_0 is the acoustic impedances of the coupling fluid, and α_c is the attenuation in the coupling fluid through a distance $2d$. The attenuation coefficient α_0 can be calculated using

$$\alpha = \alpha_0 f^n, \quad (7)$$

where a linear frequency dependence was assumed with $n=1$, similar to that of most tissue [12]. Details of how the ultrasonic properties were determined using these equations is given in [10], [11].

A. Cells

MCF-7 breast cancer cells (ATCC, VA, USA) were used in all experiments. Cells were incubated at 37°C with 5% CO₂ in Dulbecco's modified eagle's medium (DMEM, ATCC, VA, USA) with 10% fetal bovine serum (FBS) and 0.1% insulin. Cells were passed every 3 days to maintain exponential growth. Cells were transferred to Lab-Tek II chamberslides (Nunc, Germany) and allowed to incubate for 24 hours. For apoptotic measurements, the medium was replaced with DMEM without FBS or insulin, and 3 mg/mL caffeine and 20 ng/mL paclitaxel were added to induce apoptosis [13]. Cells were incubated for 24-72 hours prior to measurement.

Thirty minutes prior to experimentation, stains were added to help identify mitotic and apoptotic cells. Hoechst 33342 labels DNA. The morphology of chromosomes inside the cell was used to identify the cell cycle during mitosis. Annexin-V and Propidium Iodide was added to identify cells undergoing early and late stage apoptosis. Positive Annexin-V only indicate early stage apoptosis, while positive Annexin-V and positive propidium iodide indicate late stage apoptosis. Cells were then transferred to the acoustic microscope system, which was housed in a climate controlled box maintaining a constant temperature of 36°C.

B. Acoustic Microscope

The SASAM 1000 acoustic microscope (Kibero GmbH, Saarbrücken, Germany) was used for all measurements. It combines an Olympus IX81 inverted microscope with a rotating acoustic module containing the transducer positioned above the optical objective. The sample was positioned between the optical objective and transducer, allowing for simultaneous optical and acoustical measurements.

Transducer movement is controlled using a piezoelectric system (Piezosystem Jena GmbH, Jena, Germany) which has a precision of 0.1 μm in the x , y and z direction. The transducer is scanned over the sample using a raster pattern using step sizes ranging from 0.1 to 2 μm , with up to 1000 a-lines averaged at each point. The signal is amplified by a 40 dB amplifier and digitized at a rate of 8 GHz. 10 Vpp pulses are generated using two monocyte pulse generators with center frequencies of 300 and 1000 MHz, each with 100% bandwidth. The pulse repetition rate was up to 500 kHz. A transducer with a 375 MHz center frequency, 60° aperture angle and -6 dB bandwidth of 42% was used for all measurements.

C. Measurements

Once a cell state was identified from staining (interphase, metaphase, apoptosis), $V(z)$ measurements were made over the central region of the cell using the 375 MHz transducer. A step size of 1 μm was used over a range of roughly $\pm 30 \mu\text{m}$ around the substrate focus. The properties of the cells were then calculated using equations 1-7 using the time and amplitude of the resulting signals. A total of 110 cells were measured: 34 interphase cells, 21 metaphase cells, 29 early apoptotic and 26 late apoptotic cells.

TABLE I. ULTRASONIC PROPERTY CALCULATIONS

Cell State	Thickness (μm)	Sound Velocity (m/s)	Acoustic Impedance (MRayls)	Density (kg/m^3)	Bulk Modulus (GPa)	Attenuation (dB/cm/MHz)	Number of Cells
Interphase	11.0 ± 2.0	1570 ± 15	1.561 ± 0.014	994 ± 13	2.45 ± 0.03	1.44 ± 0.15	34
Metaphase	18.9 ± 1.3	1567 ± 11	1.554 ± 0.015	992 ± 9	2.43 ± 0.03	1.10 ± 0.25	21
Early Apoptotic	15.3 ± 2.4	1588 ± 20	1.554 ± 0.014	979 ± 13	2.47 ± 0.04	2.00 ± 0.45	29
Late Apoptotic	12.5 ± 2.1	1555 ± 13	1.537 ± 0.009	988 ± 7	2.39 ± 0.03	1.40 ± 0.64	26
Water	-	1521	1.521	1000	2.31	0.0014	-

The unpaired t-test was used to compare the thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation between interphase, metaphase, early and late stage apoptosis. A p -value of 0.05 or less was considered statistically significant.

IV. RESULTS

The thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation were calculated for untreated cells in interphase and metaphase, and treated cells in early and late stage apoptosis as shown in table I. The average and standard deviation for the cells measured in each state are given. The t-test was used to determine if the properties between cell states were significantly different. Histograms of the attenuation for cells in each state are shown in fig. 2.

The thickness increased from interphase to metaphase (11.0 ± 2.0 to $18.9 \pm 1.3 \mu\text{m}$, $p = 0.000$), and from interphase to early apoptosis (11.0 ± 2.0 to $15.3 \pm 2.4 \mu\text{m}$, $p = 0.000$), which is expected due to rounding of the cell during mitosis and apoptosis. The thickness then decreased from early to late apoptosis (15.3 ± 2.4 to $12.5 \pm 2.1 \mu\text{m}$, $p = 0.000$). The decrease in thickness from early to late apoptosis may be due to a decrease in cell volume typically observed during apoptosis.

The sound velocity was statistically similar between interphase and metaphase (1570 ± 15 to 1567 ± 11 m/s, $p = 0.395$). However an increase in sound velocity was observed between interphase and early apoptosis (1570 ± 15 to 1588 ± 20 m/s, $p = 0.0002$), then a decrease from early to late stage

apoptosis (1588 ± 20 to 1555 ± 13 m/s, $p = 0.000$).

The density was statistically similar between interphase and metaphase (994 ± 13 to $992 \pm 9 \text{ kg/m}^3$, $p = 0.505$). A decrease in density was observed between interphase and early apoptosis (994 ± 13 to $979 \pm 13 \text{ kg/m}^3$, $p = 0.000$), then an increase from early to late stage apoptosis (979 ± 13 to $988 \pm 7 \text{ kg/m}^3$, $p = 0.002$).

There was a small, but statistically significant decrease in the bulk modulus by 0.02 GPa between interphase and metaphase (2.45 ± 0.03 to 2.43 ± 0.03 GPa, $p = 0.021$), and increase by 0.02 GPa between interphase and early apoptosis (2.45 ± 0.03 to 2.47 ± 0.04 GPa, $p = 0.030$). The largest change in bulk modulus was from early to late apoptosis (2.47 ± 0.04 to 2.39 ± 0.03 GPa, $p = 0.000$), a change of 0.08 GPa. A study using atomic force microscopy (AFM) to measure the Young's modulus reported a decrease in the Young's modulus from untreated to apoptotic leukemia cells [14]. The Young's modulus (E) and bulk modulus (K) are related through the equation

$$E = 3K(1 - 2\nu)$$

where ν is Poisson's ratio. Assuming that a cell is homogeneous and isotropic (a reasonable first approximation given the axial and lateral resolutions of the transducer at 375 MHz are $4 \mu\text{m}$), and increase in the Young's modulus suggests an increase in the bulk modulus if the Poisson's ratio remains constant. It should be noted though that the AFM measurements do not probe the mechanical properties at the high frequencies used in this work.

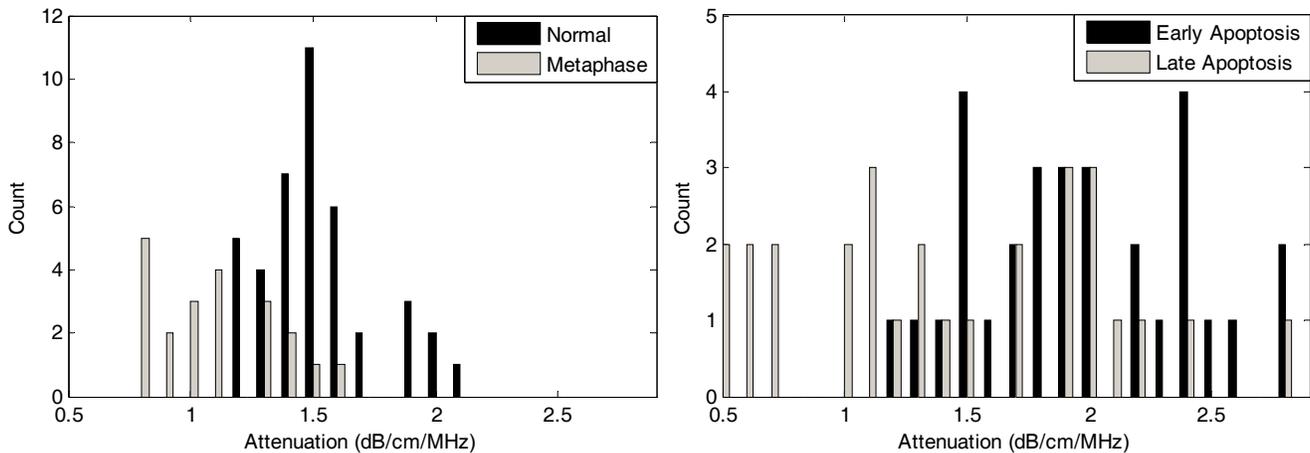


Figure 2. Attenuation histograms for normal and metaphase (left) and early and late stage apoptosis (right).

The attenuation decreased from interphase to metaphase (1.44 ± 0.15 to 1.10 ± 0.25 dB/cm/MHz, $p = 0.000$). The attenuation increased from interphase to early apoptosis (1.44 ± 0.15 to 2.00 ± 0.45 dB/cm/MHz, $p = 0.000$), but then decreased from early to late apoptosis (2.00 ± 0.45 to 1.40 ± 0.64 dB/cm/MHz, $p = 0.0003$).

The late apoptosis attenuation measurements varied from 0.4 to 2.4 dB/cm/MHz (fig. 2), the largest range compared to cells in other states. During apoptosis, nuclear material fragments and organelles become organized into apoptotic bodies. Measurements were recorded over the central region of the cell. It is possible that the material in this region varies from measurement to measurement during apoptosis, as we have measured rapid temporal variations in the backscatter signals during apoptosis [11]. The attenuation could change depending on the structures present within the ultrasound beam width during the time of the measurement.

V. CONCLUSIONS AND FUTURE WORK

A statistically significant difference was found in many of the cellular properties from interphase to metaphase and early apoptosis, and from early to late apoptosis. This study expanded on previous research using similar techniques by using a larger sample size, and fluorescent staining to accurately identify the functional state of the cell. Future work will concentrate on measuring the properties of cells through the various stages of mitosis, different cell lines and a comparison between benign and malignant cells.

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