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Applying High-Frequency Ultrasound to Examine Structures and Physical Properties of Cells and Tissues

Frank Winterroth

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Abstract

Medical ultrasound is a diagnostic imaging technique used for visualizing subcutaneous body structures. The frequencies used in conventional diagnostic ultrasound are typically 2–10 MHz. For scanning acoustic microscopy (SAM), the frequencies applied to image cells and tissues are >50 MHz. Increasing the frequency increases spatial resolution, but reduces the depth that can be imaged. The advantages of using SAM over conventional light and electron microscopes include imaging specimens without requiring any preparations which may kill or alter them; this provides a more accurate representation of them. SAM's main components are similar to those found on typical light microscopes, but the lens is often replaced by a confocal transducer. The ultrasound signal encountering the specimen generally has three results: scatter, transmission, or reflection; these signals are then merged to form the image as either a B-Scan or C-Scan. The acoustic parameters determining the image quality are absorption and scattering. SAM can objectively quantify the surface characteristics of the specimen being scanned and can also study the elastic properties of cells and tissues to discern differences between healthy and affected conditions. SAM has the potential as a major instrument of detection and analyses in biomedical research and clinical studies.

**Keywords:** acoustics, B-Scan, C-Scan, RMS data, elastography

1. Introduction

Ultrasound imaging is an extremely broad and elaborate topic covering many facets that are too complex to be covered over the course of a single book chapter. Although this chapter is dedicated to the arena of acoustic microscopy, this topic in and of itself also entails far more

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details than can be covered here. For pedagogical purposes, the material has been condensed to introduce the basic concepts and definitions for this unique form of microscopy.

1.1. Ultrasound in imaging: definition, operation, and image types

Medical ultrasound (or ultrasonography) is a common diagnostic imaging technique used to noninvasively visualize subcutaneous body structures including tendons, muscles, joints, vessels, and internal organs for possible pathology or lesions; its most common use is in obstetrics to image the developing fetus. In physics, the term "ultrasound" applies to all sound waves with a frequency above the audible range of human hearing, approximately 20,000 Hz. The frequencies used in conventional diagnostic ultrasound—including for obstetrics—are typically 2–10 MHz [1, 2].
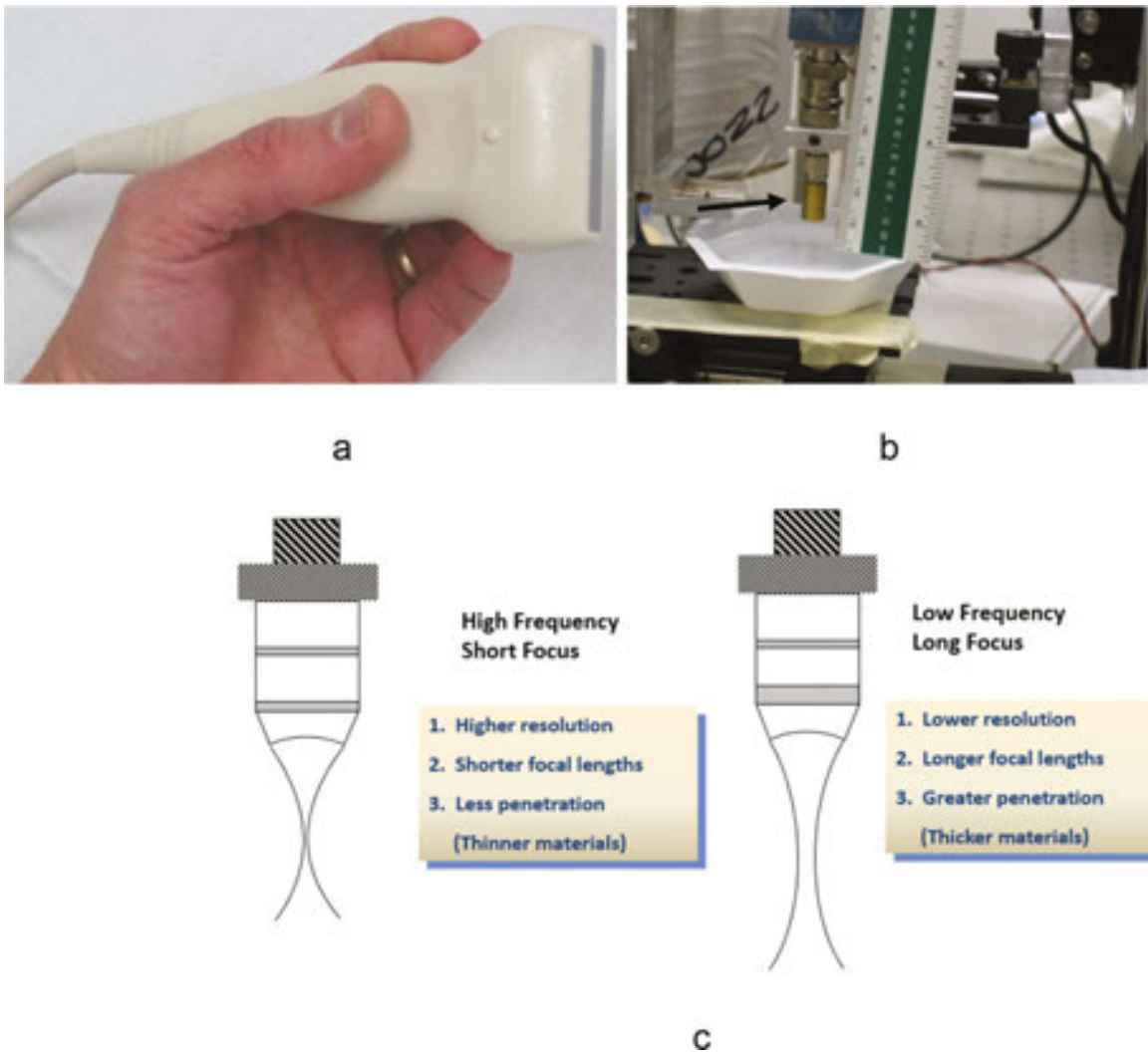
Figure 1. Comparison between a typical multielement, linear array transducer using in standard medical ultrasound, (a) and a single element confocal transducer on the SAM (b). Note the former device is handheld, while the SAM's transducer (arrow) is mounted above the specimen stage and is approximately the size of a standard pen cap. An illustration contrasting the differences between the two transducer types is included (c).

The most common type of 2D image by using conventional ultrasound is a B-Mode (brightness mode) image which is produced from the acoustic impedance (Z) of the object being scanned by the system [1, 3, 4]. The speed of sound varies as it travels through different materials and is dependent on the acoustic impedance of the material. However, the sonographic instrument assumes that the acoustic velocity (C) is constant. An effect of this assumption is that in a real body with nonuniform tissues, the beam becomes somewhat defocused and image resolution is reduced. Typical acoustic velocity in degassed/deionized water is at 25 °C = 1497 m/s.

The transducers used in conventional ultrasound imaging usually consist of a handheld device with multiple elements (**Figure 1a**)—and sometimes a servomotor—allowing simultaneous radiofrequencies which are then merged into a real-time image.

The sound from the transducer is focused either by the shape of the transducer, a lens in front of the transducer, or a complex set of control pulses originating from the ultrasound scanner (beamforming). This focusing produces an arc-shaped sound wave from the face of the transducer. The wave travels into the body and comes into focus at a desired depth.

The return of the sound wave to the transducer results in the same process as sending the sound wave, except in reverse. The returned sound wave vibrates the transducer which converts the vibrations into electrical pulses that travel to the ultrasonic scanner where they are processed and transformed into a digital image.

To produce an image, the ultrasound scanner must determine two factors from each received echo:

1. The difference in the time it takes for the echo to be received from the time when the sound was transmitted.

2. The intensity of the echo.

Once the ultrasonic scanner determines these two factors, it can locate which pixel in the image to light up and to what intensity.

Transforming the received signal into a digital image may be explained by using an analogy involving a long, column-like, multilayered box. Picture placing a transducer at the top of the box and then sending pulses down the length of the box; listen for any return echoes. When an echo is heard, how long it took for the echo to return is noted; the longer the wait, the deeper the layer (1, 2, 3, etc.). The strength of the echo determines the brightness setting for that layer (white for a strong echo, black for a weak echo, and varying shades of gray for everything in between). When all the echoes are recorded for the box, a grayscale image can be produced for it.

To generate an acquired 2D ultrasound image, the ultrasonic beam is swept across the area being examined. A transducer may sweep either mechanically by rotating or swinging it manually; a 1D phased array transducer may also be used to sweep the beam electronically. The received data is processed and used to construct the image. The acquired image is then a 2D representation of the slice into the body.

3D images can be generated by acquiring a series of adjacent 2D images. Commonly, a specialized probe that mechanically scans a conventional 2D image transducer is used. However, since the mechanical scanning is slow, it is difficult to make 3D images of moving tissues. Recently, 2D phased array transducers that can sweep the beam in 3D have been developed. These can image faster and can even be used to make live 3D images of a beating heart [5].

Ultrasound also applies Doppler sonography in order to study physiological processes such as blood flow and other organ functions [1, 2]. The different detected speeds are represented in color for ease of interpretation. To image and differentiate tissues and organs—in particular healthy and affected—the damaged tissue often shows up in a unique color (i.e., flash of different color in a blood vessel leak). Colors can alternatively represent differences in amplitudes between any received echoes. Doppler ultrasound is generally applied in conventional ultrasound, but not in acoustic microscopy; it will not be discussed in detail here.

1.2. Acoustic microscopy: background, specifications, applications, and advantages

The scanning acoustic microscope (SAM) has been employed since the 1970s—mostly in materials sciences and engineering—typically to study failure analyses and nondestructively assess structural properties. In the past two decades, it has been similarly utilized in biology and medicine to examine the structures and functions of cells and tissues [18]. For SAM, the frequencies applied to image materials are usually greater than 50 MHz; this provides a significant dynamic range ratio between the lowest and highest values used in ultrasonic.

In contrast to the aforementioned conventional ultrasound system, the acoustic microscope often employs a smaller transducer—similar in size to a pencil eraser—mounted on the body of the microscope (**Figure 1b**). The SAM's transducer is generally a single-element piezoelectric confocal unit, producing a signal similar to its conventional counterpart.

Given such high frequencies for imaging, there is a trade-off between penetration depth of the signal through an object and spatial resolution. With higher frequency ultrasound, there is an increased attenuation coefficient, resulting in it being more readily absorbed by the material it is scanning. This is analogous with light microscopes: Increasing the magnification enhances a particular area, but reduces the field of view of the imaged specimen; likewise, with SAM, increasing the frequency increases spatial resolution, but reduces the depth that the specimen can be imaged. **Figure 1c** illustrates the differences between high- and low-frequency ultrasound imaging, comparing the benefits and limitations between them.

A typical SAM transducer used in imaging biological specimens is approximately 50–60 MHz. Its parameters are the following:

1. 15 μm scanning step size in both the transverse and horizontal directions.

2. Axial resolution (R_{ax}) equals 24 μm.

3. Lateral resolution (R_{lat}) equals 37 μm.

4. Depth of field (DOF) equals 223 μm.

5. The f–number is 1.5.

6. A routine scan is sampled at 300 mega samples/second.

Note: All of these parameters can be manually adjusted to accommodate a specific scan of the desired object.

The axial resolution depends on the dimensions of the pressure pulse, which is related to the transducer bandwidth (BW) and system electronics:

$$R_{ax} = \frac{c}{2 \times BW} \qquad (1)$$

where c is the sound speed in the tissue and BW is 32 MHz. Likewise, the lateral resolution at the focal point can be estimated by:

$$R_{lat} = \lambda \times f \, / \, number \qquad (2)$$

where λ is the wavelength (25 μm).

The depth of field (DOF) is calculated by:

$$DOF = 4(f \, / \, number)^2 \lambda \qquad (3)$$

Due to a small f/number (which provides a tight beamwidth), the DOF is also limited.

Lateral resolution is the resolution orthogonal to the propagation direction of the ultrasound wave.

Acoustic impedance (Z): The impedance determines the amplitude of the reflected and transmitted waves at the fluid–tissue interface. Complex scattering properties of tissues are due to acoustic impedance interfaces in microstructure of tissues:

$$Z = \rho c \qquad (4)$$

$$R = |(Z_2 - Z_1) / (Z_2 + Z_1)| \qquad (5)$$

where ρ is the density of the material, and R is the reflection coefficient.

Table 1. Logistics of the SAM operating mechanisms and principles.

R_{ax} is the resolution in the direction of propagation and is determined by the length of the ultrasound pulse propagating in the tissue; R_{lat} is the resolution orthogonal to the propagation direction of the ultrasound wave. The ultrasound signal encountering the specimen has three

possibilities: scatter where the signal travels in directions orthogonal to the transducer, transmission where the signal passes through the specimen, or reflection where the signal is received back to the transducer for processing into an image. Essentially, it is this impedance mismatch between the signal propagating through water and encountering the specimen which results in the processed image. **Table 1** provides further comprehensive details of the SAM's operating mechanisms, including axial and lateral resolutions, DOF, and acoustic impedance.

The advantages of using SAM over conventional light and electron microscopes include being able to image materials—including cells and tissues—without performing any preparations which could potentially kill or alter them. This provides a more accurate representation of the materials' natural properties [18]. In biological imaging, it can also provide evidence as to any degree(s) of differentiation which the cells undergo without chemically affecting their properties [6–9]. In some SAM systems, there is also the ability to scan across three axes, allowing for a three-dimensional composite image of the desired specimen to be produced. This is covered in greater detail later in this chapter.

2. Principal components of the scanning acoustic microscope

### 2.1. Transducer and general design layout

The SAM's main components are similar to those found on typical light microscopes. In place of the magnifying lens, there is often a single-element confocal transducer attached to stepper motors which control the transducer's movement in three dimensions: horizontal, transverse, and vertical. For the stage, there is also a small tank filled with fluid—usually degassed/deionized water—in the specimen being imaged as immersed (**Figure 2**). Just as with any conventional ultrasound, when imaging with the SAM, all materials being imaged must be submerged in a fluid medium or have coupling gel between the transducer and the area being scanned as ultrasound energy scatters when exposed to air.
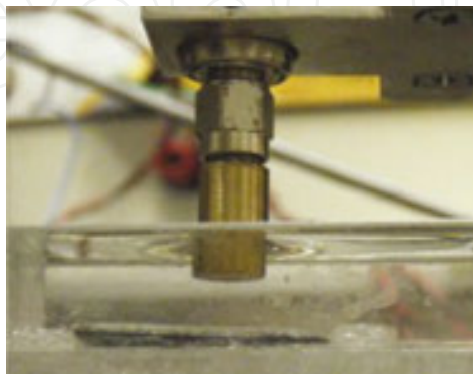
Figure 2. Close up of the SAM transducer immersed in a tank of degassed and deionized water, above the material to be imaged.

2.2. Principal features of the SAM and its operation

In addition to the stage, other key features of the SAM include the following: a broadband high-frequency pulser where the excitation signal originates; a diode expander which reduces the baseline noise before the signal travels into the transducer where it is converted into ultrasonic waves. Signals received by the transducer go into a diode limiter which blocks some of the high-amplitude excitation signal from the receiving electronics. The received signal is generally amplified by a set of attenuators and 32 dB fixed-gain amplifiers. Finally, the signal is captured by a high-speed 8-bit digitizing board.
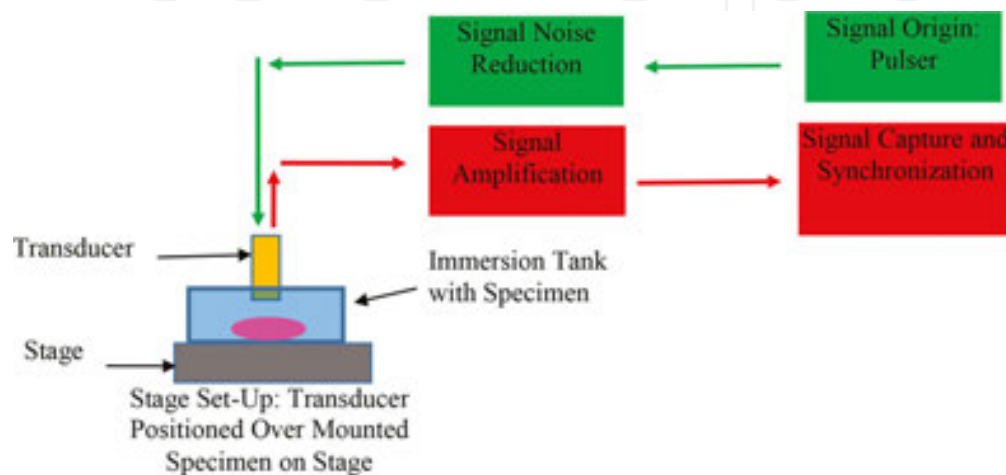
Figure 3. The principal operating units relative to the SAM's overall layout. In addition to the stage, other key features of the SAM include the following: a broadband high-frequency pulser where the excitation signal originates; a diode expander which reduces the baseline noise before the signal travels into the transducer where it is converted into ultrasonic waves. Signals received by the transducer go into a diode limiter which blocks some of the high-amplitude excitation signal from the receiving electronics. The received signal is generally amplified by a set of attenuators and 32-dB fixed-gain amplifiers. Finally, the signal is captured by a high-speed 8-bit digitizing board. The SAM signals are then synchronized to reduce any signal to signal jitter and improve the efficiency of signal averaging.

A custom-built circuit takes the 10 MHz synchronized output from the digitizing board and divides it down to a 9.76 kHz square wave that triggers both the pulser and the digitizing board. The SAM signals are synchronized to reduce any signal to signal jitter and improve the efficiency of signal averaging. A basic illustration of the SAM signal transmission and reception is shown in **Figure 3**: the principal operating units relative to the SAM's overall layout.

3. Basic operations: set-up and acquisition of standard 2D images

3.1. Specimen setup

Because the desired object for imaging is often submerged in an imaging tank (often no larger than 20 cm^3), the object itself should be no greater than 1.0 cm^2 in area (both the object's size and its scanning areas can be increased as long as the operator knows and recalls the exact region being examined is) and no thicker than approximately 5.0 mm, less than the focal length

of the transducer. Further, it must not be composed partly or completely of material that is miscible in water after being submerged for approximately 2–3 h. Ideally, the object should have greater density than the water, but with most biological materials, this is not the case. Therefore, a method of mounting the object either with push pins or embedding the base of it deep into mounting clay at the base of the immersion tank would be suitable. The area being imaged must be fully exposed to the transducer. Examples of mounting the specimens are illustrated in **Figure 4** with respect to their positions to the SAM transducer. The tank with the specimen must be placed on the stage below the transducer with enough fluid in the tank so that it is approximately 1–2 mm below the rim.
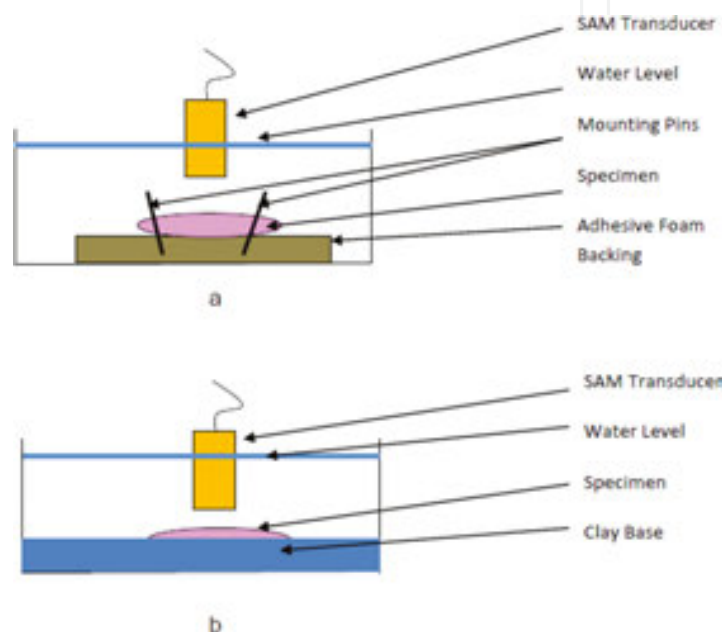
Figure 4. Two examples of mounting specimens in the immersion tank for imaging. The first involves using mounting pins through the object (away from the imaging area) into a softer backing, usually an adhesive sponge (a); the second involves embedding the specimen into mounting clay at the base of the tank, exposing the top surface of the object over the transducer (b).

3.2. Powering the microscope

The SAM requires turning on both the computer and each hardware component responsible for powering the scope. These include the power supplies for both the pulser-receiver and the digitizer. Additionally, the digitizer and pulser-receiver units must be manually powered on.

Because of the single-element configuration of the transducer—typically lacking a servomotor which allows the transducer to fluctuate—its movement is dependent on a stepper motor system. Therefore, initialization of the stepper motor driver card is imperative; this card connects to and controls the stepper motor driver.

After powering up the microscope and its accompanying computer, the operator must check the computer's appropriate site for its devices and interfaces, scroll down, and select the driver card. When the prompt screen appears, "Initialize" is selected to engage its software features.

The operator will then select an icon or tab labeled "Move"; a screen with three-dimensional axes will appear. The x-axis moves the transducer along the longitudinal direction; the y-axis moves the transducer along the transverse direction; the z-axis moves the transducer along the vertical direction. Each axis is programmed to move a specific step size (typically between 1.25 and 2.5 µm for all three axes); therefore, inputting the number of desired steps multiplies the distance of the desired step size by that number. For example: inputting 20 steps and selecting once along an axis set to 2.5 µm will move the transducer 50 µm. When operating the stepper motor, selecting "In" on the Move screen will move the transducer toward the stepper motors for each of the 3 selections, while "Out" moves it away from the motors. **Figure 5** provides a screenshot of the SAM software display for the X, Y, Z stepper motors.

Once the transducer is positioned over the prepared tank, it is slowly lowered in using the "Move" feature to control the stepper motor along the z-axis. It is recommended that once the transducer is touching the fluid surface to descend the transducer at a rate of 10–20 steps per "Move" selection. While the transducer is immersed in the fluid, but close to the surface, its bottom face is inspected under the fluid for the presence air bubbles. All air bubbles must be removed as they could scatter the ultrasound energy. Removal can often be accomplished using a cotton swab or a plastic twist tie that has a slight kink at the bottom to gently remove the bubbles off of the bottom face of the transducer; **no sharp or metallic objects must ever be applied directly to the transducer.**

Figure 5. Illustration of a typical screenshot display for the software used to operate the SAM. The gray area displays the logistics of the transducer, the specimen being scanned, and any comments about the experiment. The 3D axes x, y, and z are labeled horizontal, transverse, and vertical, respectively. The blue bars in each of the axes' fields indicate how much of each scan has been completed. The bar labeled "Pusher" refers to the compressor used for elastography.

3.3. Image acquisition

Initiation: Once the transducer is positioned over the desired area for scanning, it is necessary to obtain the RF wave of the ultrasound. This is performed with an oscilloscope and accompanying command screen. On the command screen toolbar, the operator will select the initial/recall settings; a command prompt will appear with the SAM operation commands. After selecting data acquisition commands, it is imperative to choose the acquisition modality, either "Auto" or "Normal" acquisition (one is automated; the other manually controlled): a signal wave will appear on the oscilloscope, with an acquisition rate of approximately 1000–2000/s. A trigger delay on the command window must be selected at approximately 3×10^{-6} to provide a 3.0 μsec delay; this prevents the scan from "freezing" during the signal acquisition off of the specimen. The operator then selects the appropriate channel of the acquisition software which will provide a single band for the signal. This is followed by selecting "Connect Data Points" under the "Tools" command which will enhance the signal coming off of the oscilloscope. At this point, there will appear a single rapid signal moving from right to left on the oscilloscope (**Figure 6a**).
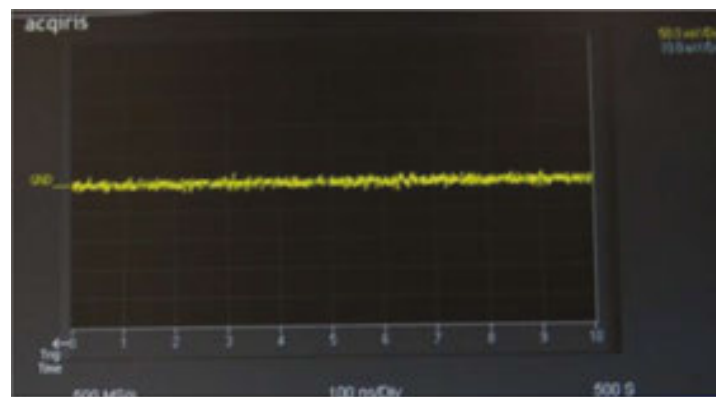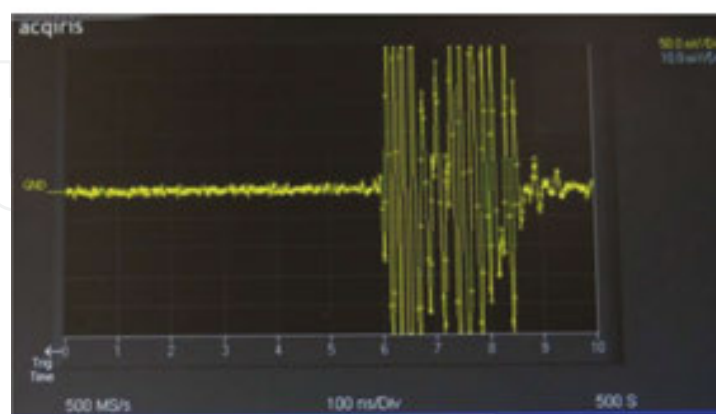

Figure 6. Oscilloscope images showing the background signal of the SAM in water (a) and upon encountering the specimen (b). The RF signal is generated by the impedance (Z) mismatch between the ultrasound energy in water and the specimen.

Focusing the transducer: The operator now restores the positioning functions of the transducer, namely the z-axis, moving the transducer in the "Out" direction—away from the stepper motor and toward the specimen—in increments of approximately 10 steps. Upon the ultrasound signal encountering the specimen, an RF signal wave appears on the right side of the oscilloscope (**Figure 6b**). At this point, the transducer's vertical position is adjusted so that the RF signal is in the center of the oscilloscope. It is imperative to pay attention to both the distance that the wave moves and to check the transducer's position relative to the object in the tank. If the signal does not appear very strong, the attenuators can be adjusted, by clicking first on the receiving attenuator, then the transmitting one; the lower the number, the greater the signal should increase. However, the trade-off is that there will be an increase in signal noise as both the low and high pass filters are being reduced. The signal is continuously adjusted until it is centered. Once this point on the oscilloscope is recorded, the signal is adjusted further until it attenuates. Here, it is best to zero out the position of the transducer in the vertical position, followed by reversing the adjustments made (by selecting "In" for the z-axis) until the signal returns back to the right edge of the scope. The operator records the distance travelled under the z-axis which is the approximate thickness of the specimen.
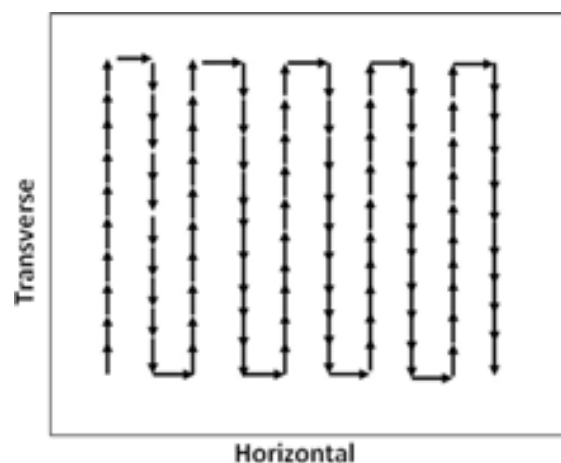
Figure 7. Top-down view illustrating the scanning pattern made by the SAM transducer. Upon scanning in the transverse direction, the transducer shifts over in the horizontal direction and the scanning process repeats itself. After the desired area is scanned, the transducer shifts in the vertical direction in order to scan multiple layers of the specimens as desired.

Once all the configurations are in place, the start key can be selected; the scope will start scanning the desired area of the specimen. A typical scan using the parameters listed above will take approximately 20–30 min. It is recommended that the SAM computer not be used during the scanning session as this often will disrupt the scan and may potentially shut down the system, which will require reconfiguring the SAM set-up. The transducer will acquire the specimen image first along the transverse line, firing 31 scans into the specimen before moving to the next point which will be 1.25 μm × the number of steps which were entered for the scan. After scanning the desired distance along the transverse axis (usually 1.0 mm), the transducer will shift 2.5 μm × the number of selected steps in the horizontal direction and repeat scanning in the transverse direction, followed by a repeated shifting 2.5 μm × the number of selected

steps in the horizontal direction and repeating in the transverse direction. It will form a "zigzag" pattern until it has completed the scan over the desired area of the specimen surface. The transducer will then shift 2.5 μm × the number of steps down in the Z direction and the process will repeat itself; **Figure 7** illustrates this scanning pattern.

After the scan is complete, both the dataset and logistics on the scan will be produced (an example of the latter is shown in **Table 2**). These are generally saved under preselected LabVIEW files.

31	Number of averages
256	Number of points per trace
923	Offset from the trigger
6	Number of 2.50 μm pulses between horizontal sampling points (X)
12	Number of 1.25 μm pulses between transverse sampling points (Y)
48	Number of 2.50 μm pulses between vertical sampling points (Z)
10	Number of 1.5875 μm pulses between compressor sampling points
70	Number of sites along horizontal axis (X)
70	Number of sites along transverse axis (Y)
7	Number of sites along vertical axis (Z)
1–5	Number of sites along compressor
0.50	Voltage scale (V)
14	Initial setting for transmission attenuator
14	Initial setting for reception attenuator
0.00	Voltage offset (V)
250	Sampling frequency (MHz)
3.000	Trigger level (V)
External	Trigger source
Positive	Trigger slope
9.76	Avtech repetition rate (kHz)
8	Avtech frequency setting (1–10)
7	Avtech amplitude setting (1–10)
1.555	F–number of the transducer
3.111	Focal length of the transducer (mm)
61.0	Center of frequency of the transducer (MHz)
30.0	Bandwidth of the transducer (MHz)

Table 2. Example of the settings and operating parameters applied to perform a standard SAM scan.

Shut down: Once the scan is complete, the transducer will reposition itself back to its original starting point. After completing all scans, the operator restores the positioning functions and raises the transducer out of the tank. The specimen can then be removed from the tank and handled in the appropriate manner. The scan is saved under the files of the scan program. The operator can go to the data folder (such as in LabVIEW), select the files from the scan, and copy and paste them into a flash drive. To power off the SAM, it is a matter of turning off all the power supplies to the SAM and shutting down the computer.

3.4. Image processing

Upon completing the specimen scan, the RF data is first translated into an acoustic envelope for each scan point. The raw acquired signal initially includes its inverse image; this is removed by eliminating all frequencies below zero frequency. An inverse Fourier transform is applied to convert the data from a frequency domain into a time domain. **Figure 8** is the resulting acoustic envelope of a processed signal; in this case, a threshold is set approximately 20 dB above the background noise so that the acoustic energy encountering the specimen will be displayed as the acoustic envelope.
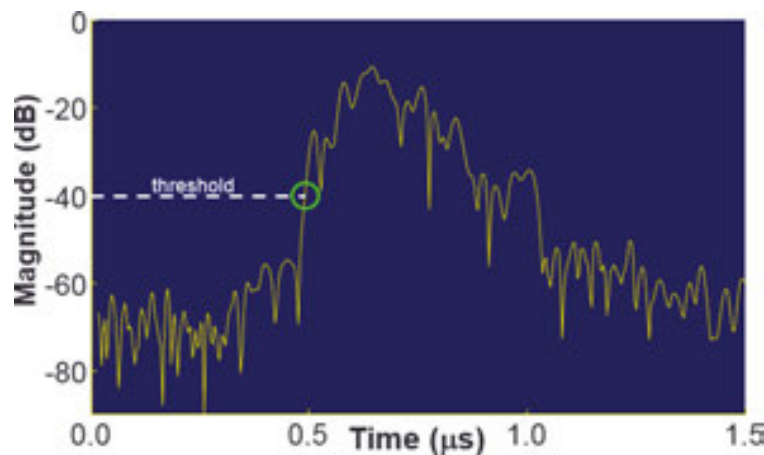

Figure 8. Representation of a typical acoustic envelope developed from the SAM scan of a specimen and derived from the raw RF scan. The raw scan initially includes an inverse image below zero frequency; this is eliminated by removing any frequency below zero. An inverse Fourier transform is performed converting the scan from a frequently domain to one of time. A threshold is set 20–30 dB above the water signal to establish point at which the acoustic signal encounters the specimen. By examining the envelope, one can see at what time the signal attenuates, allowing the calculation of the specimen's thickness.

The signals are then merged to form the image in B-Mode—called a B-Scan—which is an amalgamation of the intensities of the echoes received from the transducer. The two acoustic parameters determining the image quality are absorption which indicates how ultrasonic energy was converted into heat in the specimen, and scattering, specifically backscatter. Scattering means that an ultrasonic wave when striking an obstacle radiates part of its energy orthogonally to the specimen. Quantifying the degree of backscatter from small particles or perturbations in tissues and other materials allows differentiations in the degrees of pixel

intensity in the B-Scan images; such scatter properties include the average scatterer (energy scattered) diameter and average scatterer concentration [19].

Template 1 illustrates a program to acquire the image and converge the images using a program (such as in MATLAB) to assist in processing the acquired images from the SAM scans.
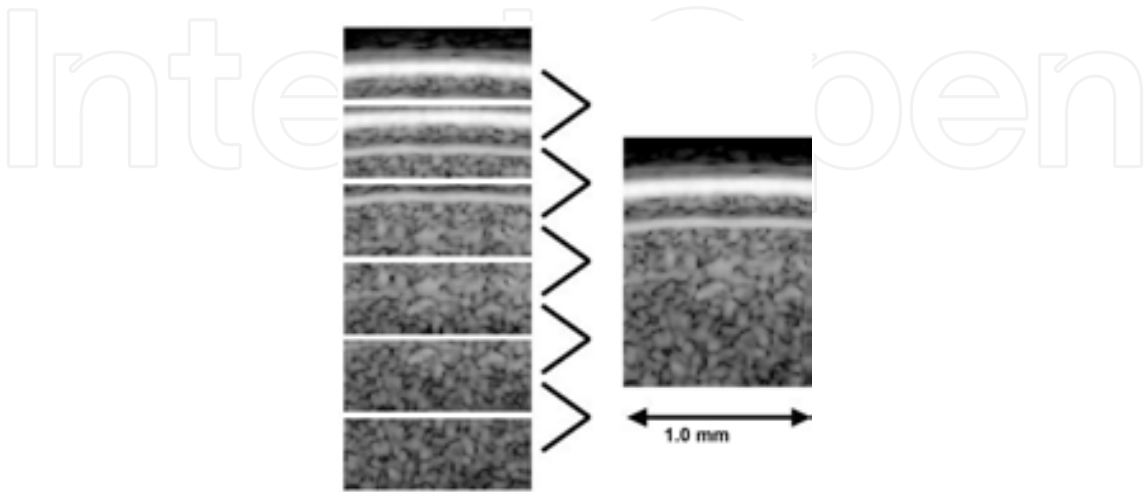
Figure 9. B-Scan merge processing in either the vertical/horizontal plane or the vertical/transverse plane. On the left are unmerged images. On the right is the resulting merged image.
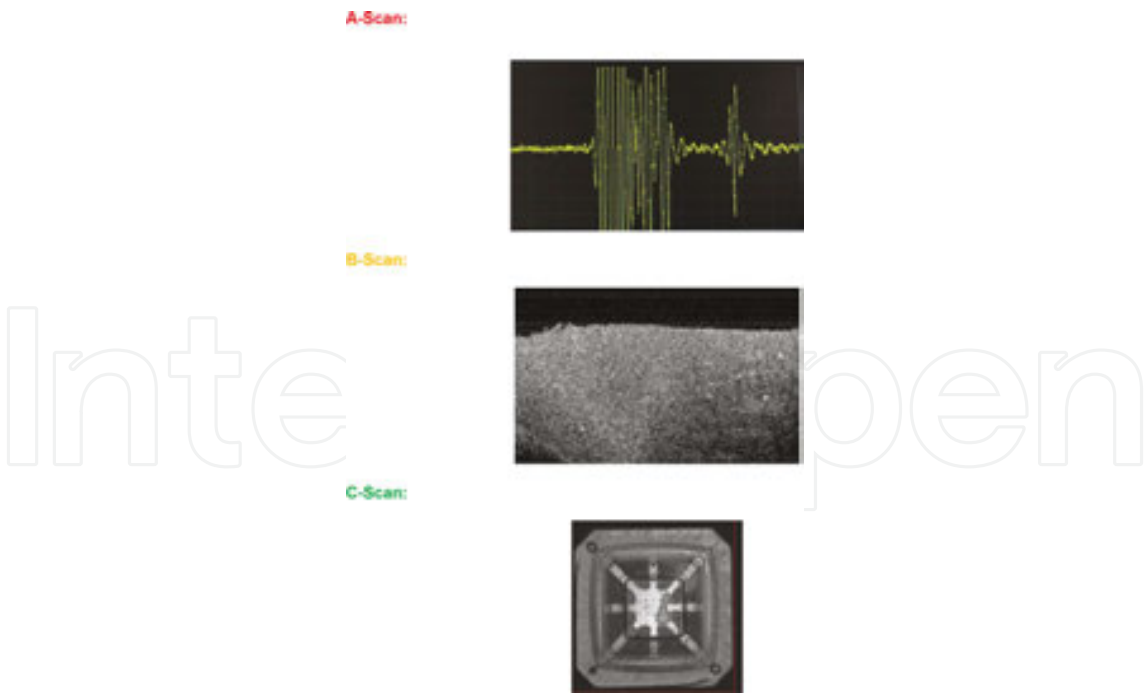
Figure 10. The "ABC's" of acoustics. Defining the raw ultrasonic signals and how they are converged to form images. The A-Scan (or A-Line) is the RF signal from a single point (x, y) in the scan. After they are converted into acoustic envelopes and merged into a vertical cross section, they form a B-Scan. The C-Scan is the collected data from a specified depth over the scanned area (a horizontal cross-section).

Table 2 provides an example of scanning logistics. It includes the number of sampling points per scan, the distances traveled by the transducer in all three dimensions, and the parameters set both manually and automatically regarding the scan. **Figure 9** shows the resultant B-Scan after acquiring and merging the scanned data. Each of the bright ovoid areas shown in the image represents scatter, and each is approximately 20–40 μm in diameter; this is within size range of many structural cells and white blood cells, but not red blood cells which are generally <10 μm in diameter. At 50–60 MHz, SAM can typically image a collective group of cells and tissues, similar to the resolution of histology images seen at 20× magnification. **Figure 10** shows comparative images of histology specimens taken with standard light microscopy (**Figure 10a** and **b**) and their SAM B-Scan counterparts (**Figure 10c** and **d**). The light microscope images were from a standard H & E stain imaged at 20×, showing the differences in surface characteristics between two biological tissues. The SAM B-Scan images were imaged using a 60 MHz transducer of similar biological tissues.

Development of higher-frequency transducers with accommodating digitizing boards have resulted in an acoustic resolutions of subcellular components, in particular noninvasive examination of cells undergoing apoptosis [3, 7].

# 4. Advanced operations: C-Scans, RMS profiles, and elasticity studies

4.1. Generating C-Scans and RMS data

The C-Scan is the scan along one selected horizontal-transverse plane of the specimen [4]; this is usually the surface of the specimen. It is similar to the B-Scan, but the merged data is orthogonal to those in a typical B-Scan. To acquire the C-Scan, the operator will enter a series of commands into a program such as the MATLAB function shown below after merging the acquired data from the scan (following acquisition of the B-Scan). This command will fit the planar surface and remove any tilt to the image, thus correcting for any irregularities stemming from incorrect mounting of the specimen.

Many of the commands contained in this chapter are custom written MATLAB functions (and some custom subfunctions within those functions). SAM operators will need the custom functions as well as the data files and MATLAB will need to be aware of the folder where the custom functions are. Template 2 is an example of MATLAB commands for generating C-Scan images

Figure 11 illustrates the three basic scanning modes in SAM as the "ABC's" of acoustics.

The C-Scan both profiles and quantifies any surface irregularities of the scanned specimen. This is done by fitting the planar image of the specimen, then subtracting everything above and below that plane. All images at the plane will appear as green; those above it will be orange-red and those below as blue. A typical C-Scan image may appear as **Figure 12**. The resulting image and root-mean-square (RMS) value will be the C-Scan profile. The smaller the number, the less surface variations there are on the specimen.
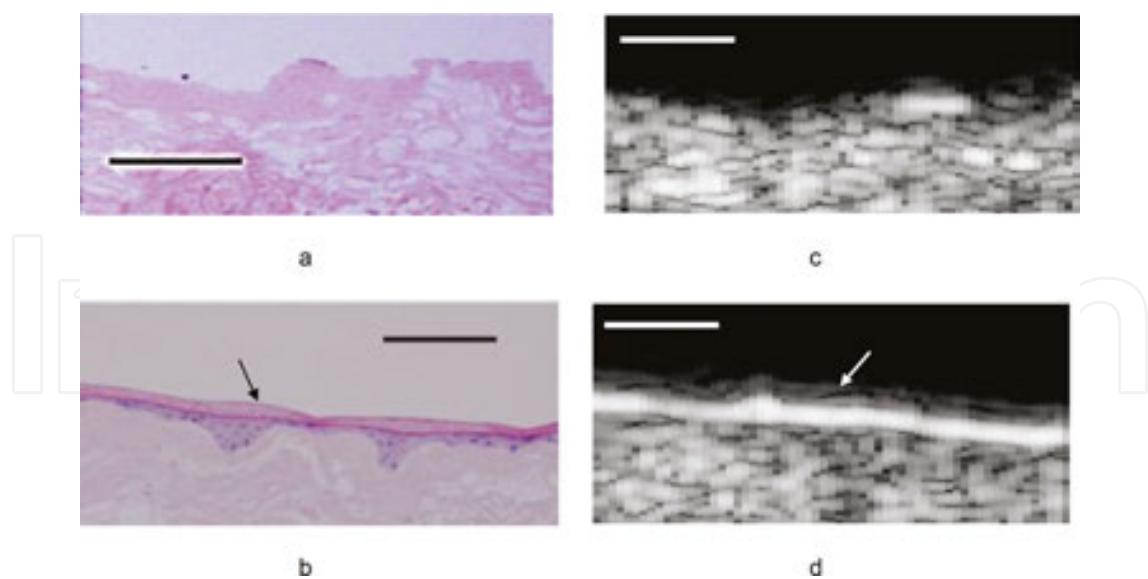
Figure 11. Comparative images of histology specimens taken with standard light microscopy (a and b) and their SAM B-Scan counterparts (c and d). The light microscope images were from a standard H&E stain imaged at 20×, showing the differences in surface characteristics between two biological tissues. The SAM B-Scan images were imaged using a 60 MHz transducer of similar biological tissues. Note the differences in the surface characteristics of light microscope images and compare them with the B-Scans: The keratinized surface shown in (b) (arrow) shows up as a bright band in (d) (arrow). Scale bars equal 100 μm.
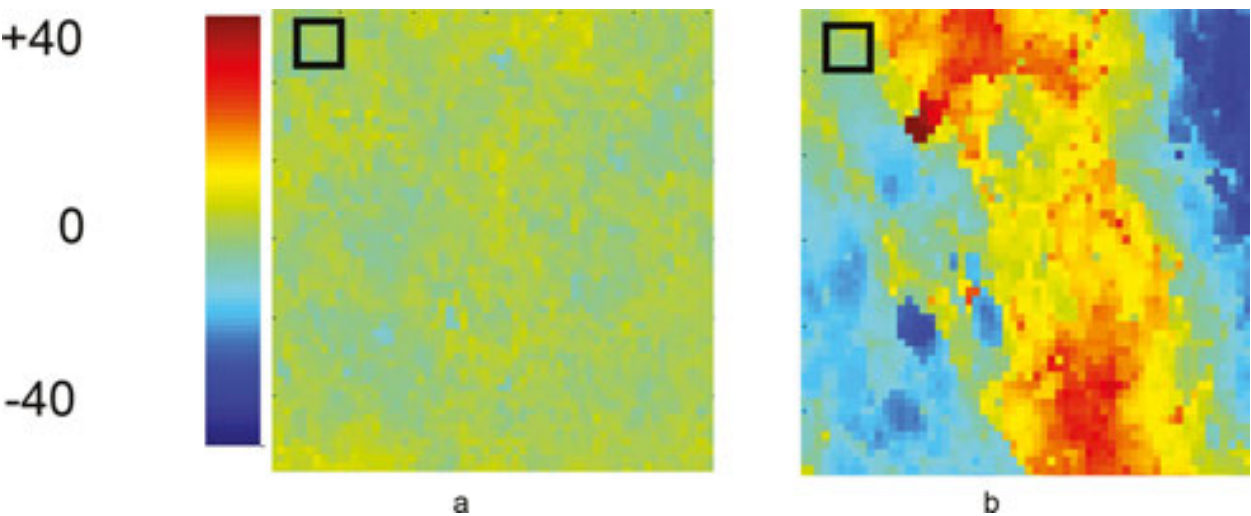

Figure 12. SAM images of C-Scans taken of two tissue specimens contrasting surface characteristics between them. One image shows the area to be essentially void of any irregularities as evidenced by its uniform color pattern (a). The second image displays significant levels of surface irregularities as the high variations in colors demonstrate. The scale boxes represent 10,000 μm². The color scale shows the degrees of variance in the surfaces, relative to their height above or below the set planar surface: The greater the yellow-green in appearance, the closer the specimen is to the planar surface and more uniform in structure. The x- and y-axes represent the number of steps in the scan along the horizontal and transverse directions; approximately 1.0 mm × 1.0 mm.

4.2. Generating 3D images

The 3D images are composites of the 2D B-Scans produced. They are generated by simply compiling and merging the acquisition data multiple times. Provided that the parameters listed under the SAM set-up procedures have been maintained, extra steps can now be taken to produce an image of the complete region that the transducer scanned.

> Template 3 is exemplifies what can be generated using MATLAB to assist in processing the acquired images from the SAM scans, following acquisition of the B-Scans, C-Scans, and RMS profiles.

An example of a complete 3D composite produced with SAM (1.0 mm^3 scan) is shown in **Figure 13**.
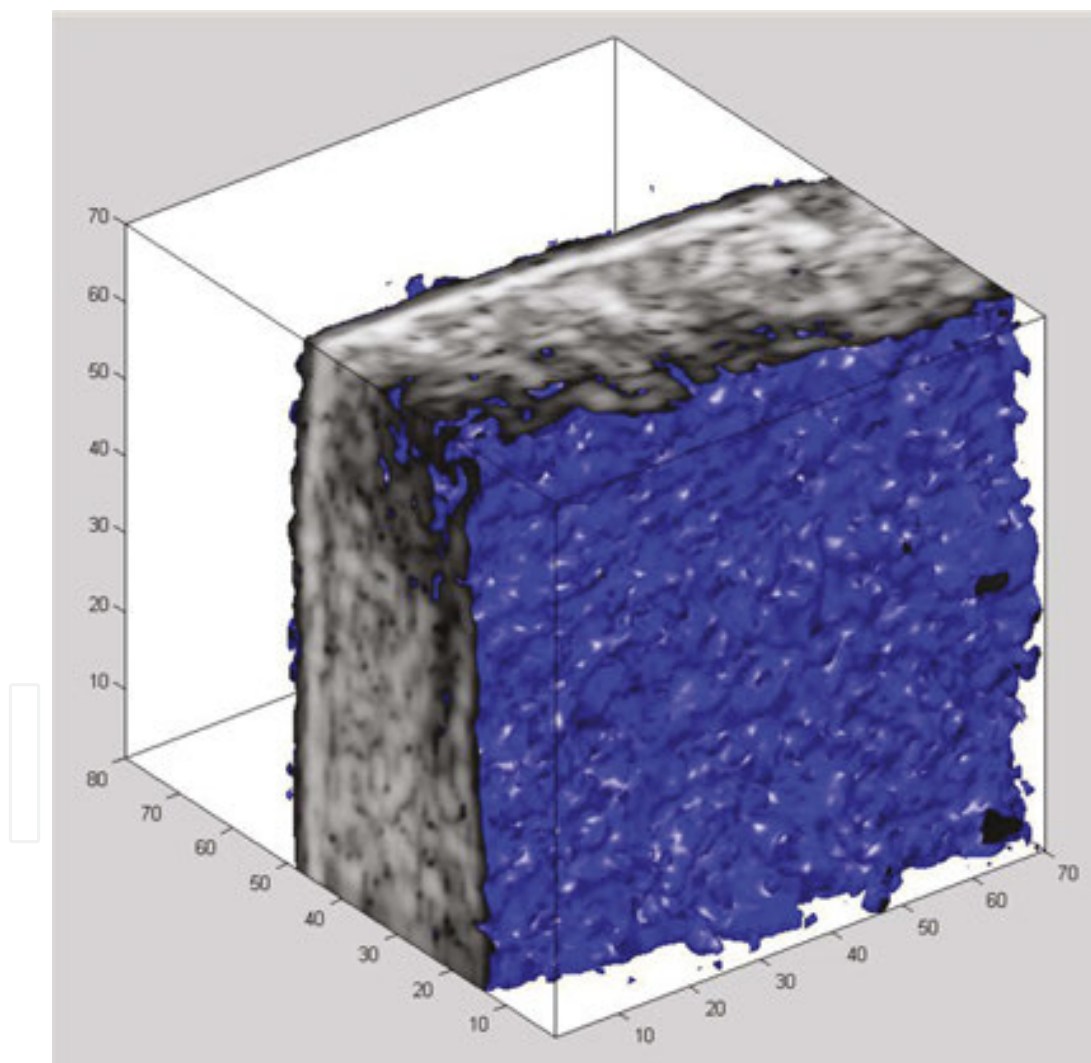
**Figure 13.** 3D rendering of a 1.0 mm^3 SAM scan of a tissue specimen. The apical surface faces forward and is rendered in blue to signify the threshold detected by the SAM transducer. Any signal detected above the threshold is rendered in grayscale: The greater the backscatter properties of the ultrasound at the point of detection, the brighter the image at that point. Any signal below the threshold is not registered.

## 4.3. Elastography (ultrasound elasticity imaging)

Ultrasound is also used for elastography, which is a relatively new imaging modality that maps the elastic properties of soft tissue [10, 11, 20]; this modality emerged in the last two decades. Elastography is useful in medical diagnoses as it can discern healthy from unhealthy tissue for specific organs/growths. For example, cancerous tumors will often be harder than the surrounding tissue, and diseased livers are stiffer than healthy ones [10–13].

There are many ultrasound elastography techniques [13]. The most prominent include quasistatic elastography/strain imaging, shear wave elasticity imaging (SWEI), supersonic shear imaging (SSI), acoustic radiation force impulse imaging (ARFI), and transient elastography. The steadily growing clinical use of ultrasound elastography is a result of the implementation of technology in clinical ultrasound machines [20]. Currently, an increase of activities in the field of elastography is observed demonstrating successful application of the technology in various areas of medical diagnostics and treatment monitoring.
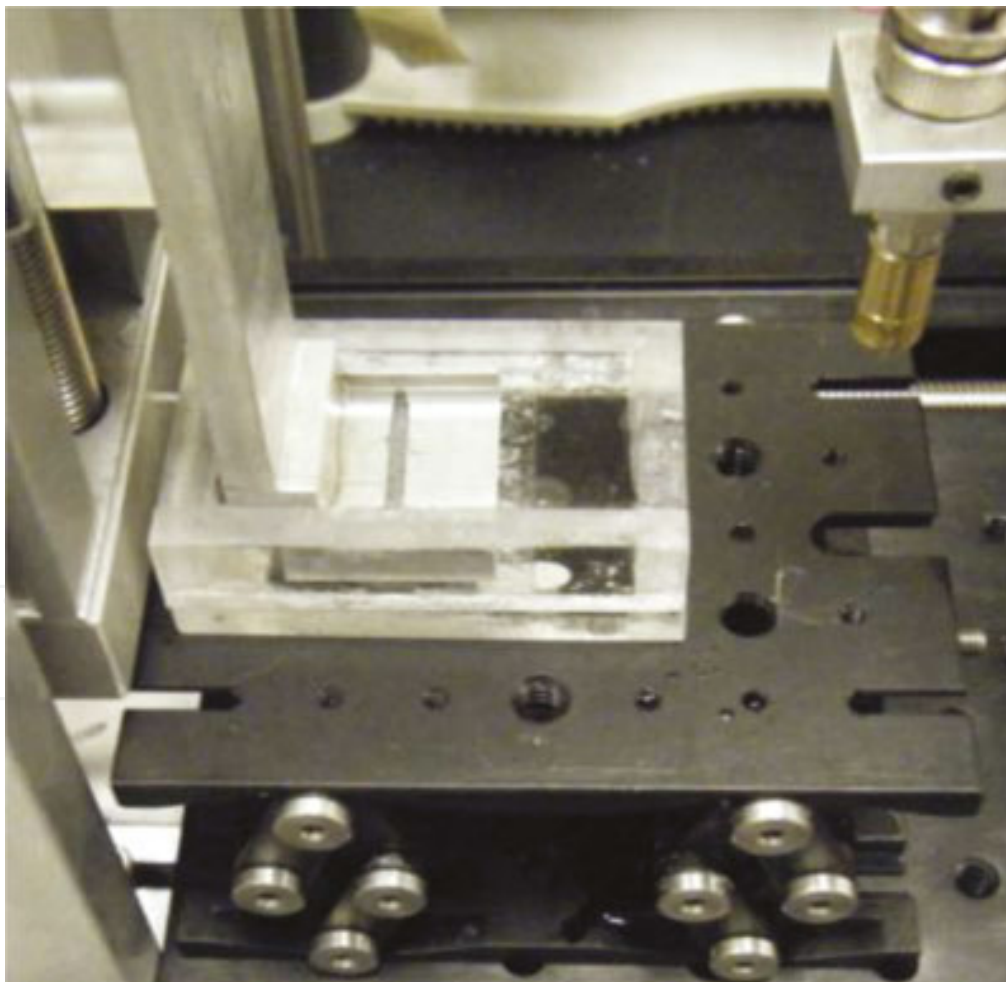
Figure 14. The SAM compressor positioned within the imaging tank. The transducer is seen on the upper right as it is being moved over the compressor.

An example of a SAM compressor is shown in **Figures 14** and **15**, by itself and in use with the transducer, respectively. The compressor has a flat aluminum plate with a slit in the center which is approximately 20 mm in length and 2 mm in width. After each scan volume is completed, the plate will move down a designated distance, compressing the specimen as the scan is repeated by the SAM. A full 3D rendering of the scanned specimen at each compression step can then be produced to determine the degree of strain at specific regions of the specimen. Here, the SAM is potentially advantageous over a conventional mechanical compressor in determining what properties in the specimen contribute to the greatest or least changes in overall strain behavior [14, 15]. In this latter case, they can be analyzed through a process called speckle tracking which is not covered in this chapter.
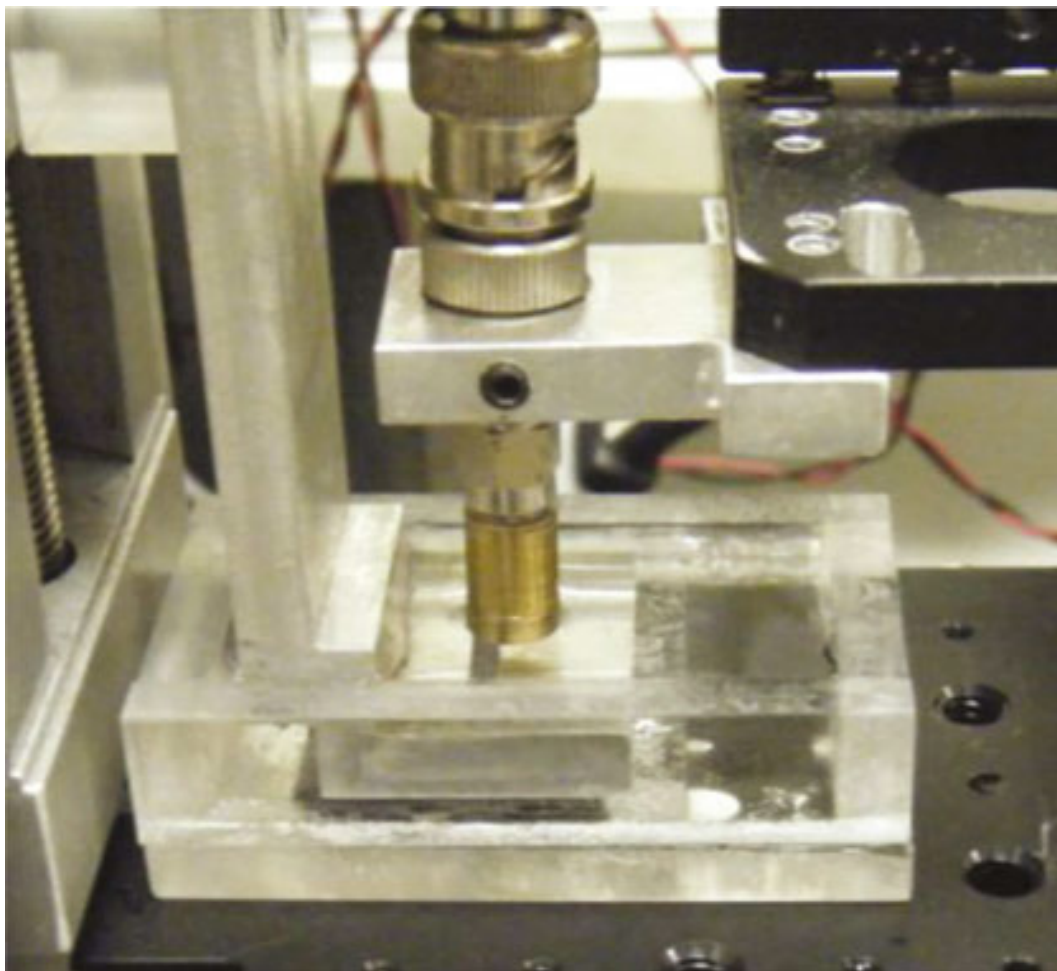
Figure 15. The SAM set up for performing compression studies. The compressor is positioned over the specimen in the tank, while the transducer is located over the slit in the compressor.

4.4. Specimen set-up and positioning

The specimen set-up for the compressor is essentially the same as described earlier with the addition of positioning the compressor plate over the object. After mounting the specimen in

the immersion tank, the operator will place the tank on the stage, add enough water to barely cover over the specimen, and slowly lower the compressor toward the tank. When the compressor plate is approximately 1.0 cm from contacting the tank, the tank is raised manually so that the compressor plate fits directly inside and over the specimen. The compressor plate can then be lowered using the "Move" program with one hand while manually lowering the tank down to the stage with the other, moved in step sizes ranging from 20 to 30. Once the tank is back on the stage, the operator continues lowering the plate until it comes in contact with the specimen, without pressing it. Once this is done, the controller axis under the "Move" program is zeroed-out. The operator can then begin moving the transducer into place so that it is directly over the compressor. *Extreme care must be taken so that the transducer is out of the way when positioning the compressor over the specimen and tank. The transducer and compressor must never contact one another*. The operator finally positions the transducer into the tank based on the guidelines described earlier.

4.5. Transducer positioning and signal acquisition

The transducer is moved in the vertical direction toward the compressor plate until a strong reflection signal is seen in the oscilloscope. At this point, the transducer should be positioned over the metallic portion of the plate—preferably to the right of the compression plate's slit. The transducer is moved in the horizontal direction toward the slit until the acquisition signal diminishes, followed by several more steps until the signal completely dissipates. The transducer will now be positioned over the compression plate slit. Care must be taken not to run the transducer into the pusher axis. There is not much room beyond the side of the slit closest to the pusher axis. The axis controller is zeroed out, and the operator slowly (in increment steps of 10–20) continues moving the transducer in the horizontal direction until the reflection signal off the compressor plate returns. Once it returns, the horizontal distance moved should read approximately 2.0 mm (the width of the slit). The transducer is moved in the horizontal direction back into the slit until it is positioned 1.0 mm in the center of the slit (the signal reading on the oscilloscope should be minimal). The transducer is now moved in the vertical direction until the acquisition signal appears again; this will be the signal reflecting off of the specimen. The transducer is moved away from the specimen again until the signal is just visible on the right side of the scope. The operator finally zeroes out the axis controller, minimizes the "Move" program, and closes out the acquisition program. The operator now follows the instructions listed under "Image Acquisition" to scan the specimen. Under "Sampling Sites," the same standards are kept as listed under the basic operations, except for the compressor.

Transverse: 70 (1050 μm); horizontal: 70 (1050 μm); vertical: 7 (960.00 μm); pusher (compressor): 1 (15.875 μm). Note that for the pusher, the number of pusher steps desired for the compression scan is selected. A typical selection ranges from 3 to 10, depending on the thickness of the specimen.

After entering the desired information, the operator starts the scan. Also note, because the compressor is now employed in the scan, the scanning program time will be multiplied by the

number of steps entered under the pusher. For instance, entering 3 steps will triple the scan time listed under "Basic Scans."

4.6. Image acquisition and generating 3D movies of the compression data

One process for producing movies resulting from the compression sequences is derived from the MATLAB files in Template 4.

The computer must have Windows Media Player—or its equivalent—in order to access the movies. Open Media Player and select the saved files, and set to continuous run, if preferred (usually, a film involving 6-step compressions runs approximately 0.5 s). The resulting images will combine to form both a movie displaying the result of the compression steps and the degrees of stiffness/compliance—based on the strain maps generated during the compressions —in the properties of the specimen being tested. The SAM can also display which stages within the specimen (if it is heterogeneous) are the greatest/least changes in the compressions. In this latter case, the operator can better correlate what specific properties within the tested specimen contribute to the observed changes.

4.7. Use of apparent integrated backscatter to quantify image surfaces

The use of apparent integrated backscatter (AIB) to quantify image surfaces further allows additional objective methods to assess the conditions of the specimen. AIB is a measure of the frequency-averaged (integrated) backscattered power contained in some portion of a back-scattered ultrasonic signal.

Changes in the material's composition will change the impedance mismatch, resulting in different backscatter characteristics. From a materials perspective, the SAM has been shown to analyze changes in material compositions, interstitial fractures, or densities within the surface and/or subsurface regions of the scanned object. From a biological perspective, we can quantify these changes and correlate them to the development of the cellular changes and production of any cellular products which result from the differentiation and evolution of the cells [5, 16].

The MATLAB commands for applying AIB to the image surfaces are exemplified in Template 5.

5. Conclusions and additional applications of ultrasound microscopy

This chapter provides a general overview of acoustic imaging techniques at the microscopic level: its uniqueness as an imaging modality along with its benefits and limitations. It also compares its principal features and differences between conventional ultrasound and light microscopy. Ideally, SAM will evolve to become a standard biomedical tool within laboratory settings—both research and clinical—as with other currently available microscopy systems.

Because SAM requires no preparatory work on the specimens, recent studies have been performed to image cells and tissues under aseptic conditions to effectively monitor their

growth and differentiation [9]. Other potential applications include real-time imaging of cells and tissues and even *in vivo* monitoring of tissues, provided there are materials and methods to image the selected tissues within the animal or person.

One possible clinical application has been recently examined: utilizing SAM's elastography abilities to test for physical properties in the cornea. This has a strong potential for improving current screening methods in patients electing to undergo LASIK surgery [16]. It may also be utilized to screen for other vision disorders, including glaucoma and keratoconus.

Another major arena of clinical study for SAM is in dermatology [6]. Because skin is an exposed organ, it is relatively easy to noninvasively examine its structures and functions, both *in vitro* and *in vivo*. SAM's elastography applications can be further applied to study strain maps within different skin specimens, to properly assess how the skin ages, what conditions are affecting it, and detecting healthy skin from pathological.

Differentiating normal versus affected conditions is a major advantage of SAM, especially in examining changes in elastic properties of cells and tissues. A recent study examined stiffness properties in breast tumors and correlating them to degrees of metastasis [17]. This and the aforementioned work on cell growth/development, cornea analyses for LASIK, and skin imaging clearly shows the potential of acoustic microscopy as a major instrument of detection and analyses in both biomedical research and possibly even in clinical patient care.

Appendices

Appendix 1:
List of MATLAB templates used in image processing

1. Producing the wavefield plot

fid = fopen('>filename<', 'r', 'b')

fread 'int32'

256k 9800 double

On the wavefield plot, the horizontal lines mean there is system noise

figure plot rf

Focal zone: set between 100 and 150 and pick area without ultrasound signal denoise ('>filename w/prefix.data<')

(This averages across 1000 signals and subtracts out lower level signals, eliminating much of the data associated with the horizontal lines.)

2. Prefilter "oval filter" used to remove the "side noises"—temporal and spatial frequency filters oval (applies to 2D custom filter on 3D dataset)

prefilter2('filename(n)'); the n at the end stands for denoise

(For this prefilter command, the filter is customized to the selected transducer's specifications. If a different transducer (with different specifications) is used, the new filter customized to the new transducer is needed.)

append 'f' onto the file name

(Which will now be suffixed as filename.nf)

3. Refile2

(Puts the scanned data in order: either xzy and yzx (z-axis depth always in the center)

4. Merge6x (or merge6y)

(Corrects any limit on the signal magnitude. If the signal is bad, then will ignore the correction. Typical use of a correction magnitude is 0.75. After calculating correlations, this will put out a baseband file (labeled 'bb') at the end in a transverse–vertical slice. In a 3D dataset: will compose each of the slices (there are 70, each approx. 15 m thick))

Template 1: Generating standard B-Mode images

pos=ithreshold(ab,40,1:300,0);

[xn,yn]=size(pos);

[x1,y1]=meshgrid(1:yn,1:xn);

coeff=planefit(x1,y1,pos);

tilt=coeff(1)*x1+coeff(2)*y1+coeff(3);

pos2=pos-tilt;

figure;imagescale(pos2);colormap jet;axis image;

rms1 = ((sum(sum(pos2.^2)))/(xn*yn)).^0.5

rms2 = 1480*rms1/(2*250)

Template 2: Generating C-Scan images

for nf = 1:10

bb = merge_load6('>Enter Filename Here<',nf,0,69,0);

ab = 20*log10(abs(bb));

ab1 = ab(:,0:69,0:69);

mask1 = threshold(ab1,70,20:500,0);

p1(nf) = max(max(ithreshold(ab1,70,10:500,0)));

mask2 = threshold(ab1,60,1:700,1);

```
ab2 = mask1.*mask2.*ab1;

view3d(ab2,1);

set(gca,'CLim',[90-60,90]);

fr(nf) = getframe(gcf);

clf;

end
```

Template 3: Commands for developing 3D images

```
bb = merge_load6('>Enter Filename Here<'nf,0,44,0);

ab = 20*log10(abs(bb));

ab1 = ab(:,2:42,2:42);

mask1 = threshold(ab1,70,20:500,0);

p1(nf) = max(max(ithreshold(ab1,70,10:500,0)));

mask2 = threshold(ab1,60,1:700,1);

ab2 = mask1.*mask2.*ab1;

view3d(ab2,1);

set(gca,'CLim',[90-60,90]);

fr(nf) = getframe(gcf);

clf;

end

movie2avi(10)

figure;

bb = merge_load6('>Enter Filename Here<',nf,0,69,0);

ab = 20*log10(abs(bb));

ab1 = ab(:,2:68,2:68);

mask1 = threshold(ab1,55,200:325,0);

mask2 = threshold(ab1,65,1:300,1); 30

ab2 = mask1.*ab1;

view3d(ab2,1);

set(gca,'CLim',[75-60,75]);
```

```
fr(nf) = getframe(gcf);

clf;

M(nf) = getframe(gcf);

end

fr2 = flipdim(fr,2);

fr3 = [fr fr2];

movie2avi(fr,' >Enter Filename Here<,'compression','none');

movie2avi(fr3, >Enter Filename Here<','compression','none');

movie(M,10)
```

Template 4: Generating 3D movies in MATLAB

```
/* load merged basebanded signals */

bb = merge_load6('>Enter Filename Here<',1,0,69,0);

/* convert to signal magnitude in dB */

ab = 20*log10(abs(bb));

/* find axial (time) position of surface from thresholding */

pos = ithreshold(ab,30,1:300,0);

/* calculate apparent integrated backscatter */

/* import reference spectrum (sp7) with workspace function */

ib1 = aibs(bb,sp7,pos,76,108:146);

/* subtract amplifier difference between tissue and reference */

ib1 = ib1 - 27;

/* calculate mean aibs over entire surface */

ibm = mean(ib1(:));

/* load merged basebanded signals */

bb = merge_load6('>Enter Filename Here<',1,0,69,0);

/* convert to signal magnitude in dB */

ab = 20*log10(abs(bb));
```

Template 5: Commands for apparent integrated backscatter

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