High and low frequency subharmonic imaging of angiogenesis in a murine breast cancer model

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List of Abbreviations

A-lines: Amplitude lines
AM: Amplitude Modulation
BP: Bandpass
CD31: Cluster of differentiation 31
COX-2: Cyclooxygenase-2
DMEM: Dulbecco's Modified Eagle Medium
FBS: Fetal bovine serum
FFT: Fast Fourier transform
FV: Fractional vascularity
HF: High frequency
HI: Harmonic imaging
HIS: Hue saturation intensity
LF: Low frequency
MIP: Maximum intensity projection
PBS: Phosphate buffered saline
PECAM: Platelet endothelial cell adhesion molecule
PI: Pulse inversion
RF: Radiofrequency
RGB: Red-Green-Blue
ROI: Region of interest
SHI: Subharmonic imaging
UCA: Ultrasound contrast agent

VEGF: Vascular endothelial growth factor
Abstract

High and low frequency subharmonic imaging of angiogenesis in a murine breast cancer model

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Angiogenesis is an important physiological process where new blood vessels are formed from already existing blood vessels unlike vasculogenesis, which involves formation of blood vessels de novo. It plays an essential role in growth of tumors and development of metastasis. This process involves activation, migration and proliferation of endothelial cells and is regulated by specific growth factors. Ultrasound is an ideal imaging modality that enables scanning in real-time with the help of ultrasound contrast agents. When the gas-filled, shell stabilized microbubbles of ultrasound contrast agents are insonified by an ultrasound beam, they exhibit nonlinear scattering and emit harmonic components of the fundamental signal in the received echoes. Utilizing the subharmonic component emitted at half of the fundamental frequency ($f_0/2$) provides an improved contrast to tissue ratio for ultrasound imaging of tumor angiogenesis.

This project compares quantifiable measures of tumor vascularity obtained from contrast-enhanced high frequency (HF) and low frequency (LF) subharmonic ultrasound imaging (SHI) to 3 immunohistochemical markers of angiogenesis in a murine breast cancer model.

Nineteen athymic, nude, female rats were implanted with $5 \times 10^6$ breast cancer cells (MDA-MB-231) in the mammary fat pad. The contrast agent Definity (Lantheus Medical Imaging, N Billerica, MA) was injected in a tail vein (dose: 200 µl/kg) and low
frequency pulse-inversion SHI was performed with a modified Sonix RP scanner (Ultrasonix Imaging, Richmond, BC, Canada) using a L9-4 linear array (transmitting at 8 MHz and receiving at 4 MHz in SHI mode) followed by high frequency imaging with a micro-ultrasound scanner, Vevo 2100 (Visual Sonics, Toronto, ON, Canada) using a MS-250 linear array transducer transmitting and receiving at 24 MHz in Nonlinear Contrast mode. The radiofrequency image data was filtered using an IIR Butterworth bandpass filter (11-13MHz) to isolate the subharmonic signal (from linear tissue and bubble signals). After the experiments, specimens were stained for endothelial cells (CD31), vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2). Fractional tumor vascularity was calculated as contrast enhanced pixels over tumor area for SHI digital images, while the relative area stained over the tumor was calculated from specimens. Results were compared on per ROI basis using linear regression analysis.

Out of 19 rats, 16 showed tumor growth (84%) and 11 of them were successfully imaged. HF SHI demonstrated better resolution but weaker signals than LF SHI. The strongest correlation determined by linear regression in this breast cancer model was between HF SHI and percent area stained with VEGF (r = 0.38; p=0.034), while there was a trend towards significance for HF SHI vs. CD31 and for LF SHI vs. COX-2 (r~0.31; 0.07<p<0.09).

Quantifiable measures of tumor neovascularity derived from contrast-enhanced HF SHI appear to be a better method than LF SHI for monitoring angiogenesis in a breast cancer model of murine xenografts (corresponding in particular to the expression of VEGF); albeit based on a limited sample size.
1. INTRODUCTION

1.1 An Overview

Angiogenesis is a vital physiological process. It consists of development of new vasculature from pre-existing blood vessels unlike vasculogenesis which consists of formation of the first primitive vascular plexus de novo. Angiogenesis is also an essential step in the growth of malignant tumors growing beyond 1-2 mm\(^3\) and for the development of metastases [1-4]. This process is a cascade of several events in which host endothelial cells are stimulated to obtain oxygen and blood supply for vascular-in-growth [5]. It thus provides a pathway for cancer cells to spread via the blood and lymph system [6-8].

Tumors are able to stimulate angiogenesis by directly secreting angiogenic substances or activating and releasing angiogenic compounds in extracellular matrix [9]. This process involves activation, migration and proliferation of endothelial cells and is regulated by specific growth factors [9]. Vascular endothelial growth factor (VEGF) is an important angiogenic factor that promotes the growth of tumor by forming immature, tortuous and leaky blood vessels [3]. Platelet endothelial cell adhesion molecule (PECAM-1 or CD31), a monocyte found on the surface of endothelial cell junctions is also a potential endothelial cell marker for angiogenesis [10]. Cyclooxygenase-2 (COX-2) is another molecule that may be involved in expansion and proliferation of tumor and hence could be a positive regulator of angiogenesis [11].
There is an increased interest in non-invasive imaging of tumors to monitor the process of angiogenesis so as to evaluate the response of antiangiogenic agents and therapies. Ultrasound is an ideal imaging modality which offers scanning in real-time with lower cost relative to other imaging modalities and even eliminates ionizing radiations [12]. It can provide information related to angiogenesis by measuring tumor flow and vascular volume [9]. Conventional Doppler imaging cannot visualize vessels smaller than 100 µm [13] and hence, very few ultrasound data is available on the early stages of angiogenesis where vessel size is in the range of 20-39 µm [14]. However, the use of ultrasound contrast agents improves the signal to noise ratio up to 25 dB and allow imaging of neovascularure associated with cancers [15-17]. The gas-filled and shell stabilized microbubbles of these contrast agents yield differences in acoustic impedance and compressibility relative to the surrounding tissue [13].

At higher acoustic pressure (>0.5 MPa) these ultrasound contrast agents show nonlinear behavior by producing harmonics (ranging from subharmonics to ultra harmonic) frequency components in their received echoes. These nonlinear components can be used to improve the contrast relative to the surrounding tissue while imaging [18]. Harmonic imaging is one such commercially available nonlinear imaging technique, which utilizes the second harmonic frequency component from the backscattered echoes to improve contrast visualization [18]. However, this technique suffers from accumulation of the received harmonic signal in the tissue, which subsequently reduces blood to tissue contrast [18]. Subharmonic imaging, wherein the echoes of the signal are received at half the fundamental frequency was used as a substitute to harmonic imaging, since the subharmonic signal components in the received echoes did not accumulate in
the tissue and could be used even at higher frequencies [19]. There have been several *in vitro* as well as *in vivo* studies reported that showed feasibility of subharmonic ultrasound imaging [19-22]. Further improvement in subharmonic imaging is achieved by using pulse inversion technique where two consecutive pulses are fired, with second pulse being inverted in phase with respect to the previous pulse [23]. This technique cancels out the odd harmonics arising from the linear echo components (including linear scattering from tissue) and enhances the even harmonics, thus improving the contrast detection.

The first use of subharmonic imaging at high frequency was described by Goertz et al. in 2005. Their group conducted *in vitro* studies using wall less phantoms and showed a higher contrast to tissue ratio for subharmonic imaging (26 dB vs. 5 dB) as compared to conventional harmonic imaging. They also conducted *in vivo* experiments on rabbit ear and mouse heart to demonstrate the feasibility of performing subharmonic imaging at a transmit frequency of 20 MHz [24]. Following these studies, there were more *in vitro* and *in vivo* studies conducted, that used high frequency subharmonic imaging [25-27]. However, this method (HF SHI) has not yet been employed to measure the tumor vascularity in angiogenesis. This thesis work intends to compare measures of tumor vascularity derived from subharmonic imaging with low and high frequency ultrasound to the molecular markers of angiogenesis in a murine xenograft model.
1.2 Thesis objective(s)

The objective of this thesis work is to compare two ultrasound imaging techniques (classified on their transmit frequencies) i.e., contrast-enhanced high frequency subharmonic imaging (HF SHI) and low frequency subharmonic imaging (LF SHI) and investigate which one is a better method to depict tumor angiogenesis noninvasively. Towards this objective we use a strategy consisting of three specific aims.

Specific Aim 1: To obtain ultrasound images of rat tumors using low frequency and high frequency ultrasound.

Subharmonic ultrasound imaging has been used extensively for in vitro as well as in vivo studies related to cancer [19-22]. Subharmonic imaging at high frequencies (>20 MHz) provides improved image resolution as compared to low frequency imaging and is able to depict the blood flow within microvasculature of small anatomical structures [28, 29]. However, it has not yet been used to image the tumor angiogenesis in a murine breast cancer model. We hypothesized that; using a scanner at transmit frequency of 8MHz and receiving ultrasound echoes at 4 MHz (subharmonic frequency) could provide low frequency subharmonic scans that would show tumors grown in a murine model of breast cancer and scanner at higher transmit frequency (24 MHz) would provide high frequency image scans of the same rat tumors, with higher resolution but with weaker signals and hence resulting in reduced imaging depth as compared to LF SHI.
Specific Aim 2: To optimize High frequency subharmonic ultrasound imaging.

The images acquired from the high frequency scanner would be at the same transmit and receive frequency (24 MHz). It is necessary to extract the subharmonic component from the RF signal corresponding to the RF data for the acquired high frequency scans. We hypothesized that the RF data corresponding to the high frequency image scans could be filtered to extract the subharmonic frequency component from the RF signal corresponding to the image data, so as to obtain high frequency subharmonic images of the tumors.

Specific Aim 3: To compare fractional vascularity (FV) measures obtained from LF SHI and HF SHI with FV obtained from pathological tumor specimens stained for three immunohistochemical markers.

Three immunohistochemical markers which contribute to the progression of tumor angiogenesis, vascular endothelial growth factor (VEGF), Platelet endothelial cell adhesion molecule (PECAM-1 or CD31) and Cyclooxygenase-2 (COX-2) were selected for comparison to the fractional vascularity of tumors measured by HF and LF SHI. The measures of vascularity, that will be obtained from ultrasound images and pathological tumor specimens stained for respective markers will be compared using linear correlation to find out which ultrasound technique is more significant determinant of tumor vascularity for all the respective markers. We hypothesized that; both the ultrasound methods (HF SHI and LF SHI) would provide significant results for fractional vascularity comparisons for at least one of the three immunohistochemical markers.
2. BACKGROUND AND LITERATURE REVIEW

This chapter presents background information and review of literature pertaining to breast cancer and its diagnosis, tumor angiogenesis, ultrasound as a diagnostic imaging modality for breast cancer and the use of contrast agents and subharmonic ultrasound imaging technique and a current review of high frequency subharmonic ultrasound imaging technique.

2.1 Breast cancer and diagnosis

Cancer is formed by uncontrolled change in cell differentiation. They often lead to metastasis of the transformed cells or even death. The breast cancer originates in the lobules and inner lining of ducts in the breast tissue [30]. Worldwide, it is estimated that more than one million women are diagnosed with breast cancer every year, and more than 410,000 will die from the disease, representing 14% of female cancer deaths [31]. Breast cancer is the second leading cause of death in women exceeded only by lung cancer [32]. The American Cancer society estimated approximately 39,620 US women to die from breast cancer in the year 2013 [32]. Besides skin cancer, breast cancer is the most commonly diagnosed cancer among American women. 22.9% of cancers in women are breast cancers [33]. On an average, each year, about 185,000 women in the United States are diagnosed with breast cancer and about 44,000 die from it [31]. The survival rates for breast cancer depend highly on the type of cancer and is the stage at which it is diagnosed. Detection of the cancer in early stages is very crucial, since it can prevent
metastatic spread of the tumor. While mammography is the most widely used technique for screening breast cancer, it does not always differentiate between benign and malignant masses and hence has to be supplemented by biopsies. Hence in 2002, Taylor et al. conducted a study with 761 breast masses to determine if complementary US imaging and Doppler could decrease the number of biopsies for benign masses and showed that ultrasound can be used as an adjunct technique when mammographic findings are indeterminate for the decision of biopsy or follow-up [34]. Besides having the potential for improving characterization of breast lesions - benign and malignant, ultrasound also reduces patients need to undergo breast biopsy and eliminates the risk of getting exposed to ionizing radiations.

2.2 Tumor angiogenesis

Angiogenesis is an essential physiological phenomenon of formation of new blood vessels from already existing ones. It is an essential physiological step in reproduction, wound repair and tissue response to ischemia [1]. Pathological angiogenesis (neovascularization) has been proved as an important factor in proliferation and expansion of tumor and metastasis [2].

Judah Folkman was the first to propose the role of angiogenesis in tumor growth where he laid the hypothesis that neovascularure is needed to support the growth and metastasis of tumors, and hence anti-angiogenic treatment might be an effective way to cure cancer [6]. While the tumor is in avascular stage (tumor mass<0.5 mm³), its cells receive sufficient nutrition and oxygen through diffusion. Its growth is limited during this stage
Tumor remains in dormant state (cease growing) until it can stimulate additional blood vessel growth by means of angiogenesis [2]. For further growth, more nutrition, oxygen and removal of waste products is essential for the parenchymal cells, which takes place by development of additional vascular network around the tumor. At this time tumor enters the vascular stage. In this stage, new capillaries invade the tumor and allow it to spread to the circulatory system; proceeding towards metastasis [2]. The process of angiogenesis is complex and consists of changes in balance between angiogenic and antiangiogenic factors, change in morphology of endothelial cells, release of proteolytic enzymes, migration of endothelial cells and capillary morphogenesis, reproduction of endothelial cells and microvascular differentiation [2]. This process is tightly regulated by several stimulatory and inhibitory factors and proceeds only when angiogenic factors that promote new vessel growth are predominant [3]. Owing to the fundamental role that angiogenesis plays in cancer growth and metastasis, there has been a growing interest in research related to its regulatory mechanism and clinical implications in the past three decades [4].
Figure 2.1: A figure representing basic steps involved in tumor angiogenesis. Here stage A represents the tumor in its avascular stage where it obtains its nutrients and blood supply from the host endothelial cell. Stage B represents release of angiogenic factors as the tumor starts growing beyond 1mm in size which stimulates migration, proliferation and formation of neovessels by endothelial cells in adjacent vessels. Stage C represents a newly vascularized tumor which progresses towards metastasis. Reproduced from [37].

2.2.1 Factors regulating angiogenesis

There has been a plenty of research since 1970’s to characterize different angiogenic mediators. These regulating factors are secreted by the tumor cells in the angiogenic phase, as a response to certain stimulus to signal the need for blood supply. Most of the angiogenic factors are directly concerned with endothelial cell proliferation [2]. The most potent regulators of angiogenesis which were selected for this study are vascular endothelial growth factor (VEGF), platelet endothelial cell adhesion molecule (PECAM or CD31) and cyclooxygenase-2 (COX-2).
2.2.1.1 Vascular Endothelial growth factor

Vascular endothelial growth factor (VEGF) is a cell signaling protein which plays an important role in the growth and differentiation of vascular as well as lymphatic endothelial cells. Expression of VEGF by tumor cells stimulates angiogenesis and enables them to grow and undergo metastasis thus, making VEGF an important factor in new vessel formation throughout the tumor development. The blood vessels formed by VEGF expression and without smooth muscle coating are often immature, tortuous and leaky unlike normal blood vessels [3]. VEGF induced tumor vasculature has a number of structural and functional abnormalities including increased permeability and chaotic structure. These abnormalities promote tumor growth [3]. Besides this, the expression of VEGF also promotes survival of new vasculature by preventing the new endothelial cells from undergoing apoptosis [3].

2.2.1.2 Platelet endothelial cell adhesion molecule (PECAM -1 or CD 31)

Platelet endothelial cell adhesion molecule (PECAM-1) also known as cluster of differentiation 31 (CD31) is another signaling protein found on the surface of monocytes, T-cells which form the intercellular junctions of endothelial cells [35]. Formation of new blood vessels depends on disruption of cell-cell attachments of endothelial cells, their migration and forming new attachments to promote blood flow. The PECAM-1 molecules located at the endothelial cell junctions initiate the formation of new junctions [10]. Findings by DeLisser et al. in rat and murine models of angiogenesis further suggest
that interactions of cell-cell adhesion molecules are important in formation of new blood vessels and hence angiogenesis [10].

2.2.1.3 Cyclooxygenase-2 (COX-2)

Cyclooxygenase-2 also known as Prostaglandin-endoperoxide synthase 2 is an isoform of COX which is responsible for formation of prostaglandins. The enzyme COX-2 is not normally present in cells, but is induced by pain and inflammation by intracellular messengers. COX-2 expression occurs in a wide range of preneoplastic and malignant conditions; it is localized to the neoplastic cells, endothelial cells, immune cells, and stromal fibroblasts within tumors [36]. It is expressed in 40% of colorectal adenomas and many other types of carcinomas [2]. The studies conducted by Denkert et al. indicate that COX-2 is expressed in human malignant melanoma and is also involved in regulation of the melanoma invasion [37]. This molecule may also be implicated in several mechanisms of tumor expansion, and metastatic potential such as tumor proliferation, apoptosis and angiogenesis [11].

2.3 Ultrasound contrast agents

Ultrasound contrast agents constitute of high molecular weight gas-filled microbubbles typically 1-10 μm in diameter and stabilized by a thin (nm) outer shell of lipid, protein or fatty acid. Their smaller diameters enable them to mimic the red blood cells and easily enter the capillary network [38]. Table 2.1 below shows current ultrasound contrast agents commercially available at one time. These agents can be injected intravenously (as they are transpulmonary) to improve the contrast of ultrasound
imaging by 15-25 dB and hence, increase the sensitivity of ultrasound imaging [15-17]. This increase in sensitivity is due to impedance mismatch between blood and air/gas filled inside the microbubble. Gramiak and Shah were the first to use contrast agents in 1968 and prove that the gas in these bubbles led to increased reflectivity in blood or tissue [39].

Table 2.1: Commercially available UCAs. Reproduced from [69].

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Year</th>
<th>Gas</th>
<th>Coating</th>
<th>Availability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echovist</td>
<td>Bayer Shering Pharma AG</td>
<td>1991</td>
<td>Air</td>
<td>Galactose</td>
<td>Off market</td>
</tr>
<tr>
<td>Albunex</td>
<td>Molecular Biosystems</td>
<td>1994</td>
<td>Air</td>
<td>Human Albumin</td>
<td>Off market</td>
</tr>
<tr>
<td>Levovist</td>
<td>Bayer Shering Pharma AG</td>
<td>1996</td>
<td>Air</td>
<td>Galactose</td>
<td>Off market</td>
</tr>
<tr>
<td>Optison</td>
<td>GE Healthcare AS</td>
<td>1997</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;F&lt;sub&gt;8&lt;/sub&gt;</td>
<td>Human Albumin</td>
<td>EU, USA</td>
</tr>
<tr>
<td>Definity</td>
<td>Lantheus Medical Imaging</td>
<td>2001</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;F&lt;sub&gt;8&lt;/sub&gt;</td>
<td>Phospholipids</td>
<td>Worldwide</td>
</tr>
<tr>
<td>SonoVue</td>
<td>Bracco SpA</td>
<td>2001</td>
<td>SF&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Phospholipids</td>
<td>Europe, S Korea, China, India, Hong Kong, Singapore</td>
</tr>
<tr>
<td>Imagent</td>
<td>Alliance Pharmaceutical Corp.</td>
<td>2002</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;F&lt;sub&gt;14&lt;/sub&gt;</td>
<td>Phospholipids</td>
<td>Off market</td>
</tr>
<tr>
<td>Sonazoid</td>
<td>Amersham Health</td>
<td>2006</td>
<td>C&lt;sub&gt;4&lt;/sub&gt;F&lt;sub&gt;10&lt;/sub&gt;</td>
<td>Phospholipids</td>
<td>Japan</td>
</tr>
</tbody>
</table>
When a microbubble is insonified by an ultrasound wave it undergoes alternate phases of compression and expansion. The size of the microbubble reduces in the positive half cycle and it expands in the negative half cycle of the ultrasound pressure wave. This behavior of the microbubbles is dependent on the frequency of the incident ultrasound wave and acoustic pressure amplitude [40]. However, for higher acoustic pressure amplitudes, the extent to which the bubbles are compressed during the positive pressure phase does not correspond to the extent of expansion in the negative pressure phase, which results in asymmetrical and nonlinear bubble oscillations [41].

Figure 2.2: A figure representing the acoustic nonlinear response of contrast agent microbubbles. At higher pressure incident RF acoustic fields the microbubbles do not compress in the positive pressure phase as much as they expand in negative phase thus generating a nonlinear response. As a result of this asymmetrical contraction and expansion the bubbles produce harmonics in the received echoes of the ultrasound beam. Reproduced from [68].
2.4 Nonlinear imaging with Ultrasound contrast agents

Microbubbles detection within the neovessels requires an increased sensitivity to specific microbubbles acoustic signatures and reduced sensitivity to echoes from the surrounding tissue [18]. The nonlinear behavior of ultrasound contrast agents can be utilized to enhance the contrast relative to surrounding tissue using the imaging methods described below.

2.4.1 Harmonic Imaging

The backscattered signal from the microbubbles contains second and higher multiples of the fundamental frequency. The technique of harmonic imaging uses these frequency components for ultrasound imaging which increase the contrast between the bubbles and the tissue. Conventional harmonic imaging (HI) was the first non-linear contrast enhanced US imaging technique developed, wherein, the ultrasound beam is transmitted at a fundamental frequency $f_0$ and the displayed image is reconstructed from the second harmonic component $2f_0$. The received echoes consist of a spectral overlap between fundamental and received second harmonic signals and hence they are filtered to minimize this overlap. [18]. Hence this method uses narrower bandwidth for imaging in order to make sure that the received signal can be separated from the fundamental signal. If the bandwidth of the received and the fundamental signal components overlap, they cannot be completely separated in the receive process. The narrowband transmitted pulse used in HI reduces both spatial and contrast resolution [18]. Another disadvantage in harmonic imaging is that the tissue also produces sufficient harmonic energy to be
detected by the high sensitivity and bandwidth of modern ultrasound equipment. Thus, a tissue image would always be present, even in the absence of contrast agents [18]. Furthermore, the higher attenuation of the harmonic frequencies compared with the fundamental frequency reduces imaging depth somewhat [19].

### 2.4.2 Pulse inversion Imaging

Pulse inversion was proposed by Patrick Phillips. This technique uses two RF pulses, which are fired consecutively. The second pulse transmitted is inverted in phase with respect to the first pulse. In any linear system the response of second pulse is an inverted copy of the response from first pulse and hence the sum of two responses is zero while in any nonlinear system (such as gas microbubbles) the responses will not be inverted copies and will end up in a non-zero sum. The remainder is related to the degree of nonlinearity [23]. Besides this, the signal amplitude doubles in the summation process and even the noise level reduces providing a higher signal to noise ratio [23]. This technique has also shown an improvement of 14 dB in contrast tissue ratio over fundamental imaging at a transmit frequency of 2MHz [42].

### 2.4.3 Amplitude Modulation

This technique sends two consecutive ultrasound pulses into the tissue, where the first pulse is scaled by a factor relative to the second pulse [42, 43]. The signals are received after scaling the response of the second pulse by the same factor. Subsequent
subtraction of successive echo signals yields cancellation of linear tissue signals and retains the nonlinear UCA echo signals. It was demonstrated that this technique could produce a contrast to tissue ratio of 14dB and higher at 2 MHz [42]. However there are no reports yet on the use of AM at higher transmit frequencies (>2MHz).

2.4.4 Subharmonic imaging

Harmonic imaging is limited at high frequencies because significant harmonic signal is also generated in tissues at diagnostic pressures, which degrade the contrast to tissue ratio. Hence subharmonic imaging was introduced, which was able to achieve high agent-to-tissue contrast at acoustic pressures and frequencies currently used in diagnostic ultrasound [19]. Subharmonic imaging involves receiving the transmitted echoes of ultrasound wave at half of the transmit frequency (fo/2).

According to Eller and Flynn, a free gas microbubbles is able to emit subharmonics only when the exciting acoustic signal exceeds a certain threshold pressure which is calculated by the following equation:

\[
\rho \left( \frac{1}{2} \right) = \rho(A) \times \frac{\rho}{\sqrt{4} \left[ \left( \frac{\omega}{\omega_0} \right)^2 - 4 \right]^2 + \left( \frac{6\lambda}{\pi} \right)^2}
\]

Eq1

Where \(\omega, \omega_0, \lambda\) and are the angular driving frequency, angular bubble resonance and the logarithmic decrement respectively and \(\rho(A)\) is the incident sinusoidal pressure field [26].
This pressure is minimal at twice the resonance frequency of gas bubble and depends on the elasticity and viscosity of the microbubbles [44]. Besides this, more dominant subharmonic peaks are obtained if the signals are narrowband [40]. The in vitro experimental studies carried out by Shankar et al in 1998 suggested that the ratio of subharmonic backscattered from contrast to that backscattered from tissue, is stronger than the ratio of backscattered second harmonic [19]. As a result, blood that contains contrast should be more easily detectable with respect to tissue if the sub-harmonic, rather than the second harmonic, is used for imaging. In 2007, Forsberg et al were the first to conduct subharmonic imaging studies on humans and showed that the in vivo SHI images from human breast cancer lesions improved the diagnosis of breast cancer (81% accuracy) and a trend toward significant improvement over mammography (p=0.07) unlike other methods conventional US, power Doppler ultrasound [22].

Goertz et al. compared the contrast tissue ratios for intravascular images obtained from high frequency subharmonic imaging (Tx: 40 MHz/Rx: 20 MHz) with those from standard harmonic imaging (Tx: 20 MHz/Rx: 40 MHz) using pulse inversion. The later showed an enhancement up to 10-20 dB over subharmonic imaging (with contrast tissue ratio ranging from 5-15 dB) [25]. They showed that at high frequencies particularly for second harmonic imaging the contrast tissue ratio decreases as the imaging depth and transmit pressure are increased. A better contrast to tissue ratio would enable better visualization of tumor neovessels and considering tumor neovascularity as a predictor in characterizing breast lesions, subharmonic contrast enhanced ultrasound could also be used for diagnosis of breast cancer; but at frequencies <40 MHz.
2.4.3.1 High frequency subharmonic imaging

Performing ultrasound imaging at high frequencies, increases image resolution at the expense of image penetration depth. It is currently widely used in pre-clinical imaging of small animals where high spatial resolution is needed. Typical frequencies range from 20–70 MHz (resulting in axial spatial resolution of 20–80 µm) compared with frequencies commonly used for clinical purposes in the range of 1–15 MHz (with an axial spatial resolution of 100–1500 µm) [45]. Thus micro-ultrasound used at higher frequencies (>20 MHz) significantly improves the resolution of received signals [28]. Developments in linear array technologies for transducers have enabled imaging at frequencies in the range of 15-70 MHz [46]. Ultrasound systems operating at high frequencies not only depicted small anatomical structures [47], but were even able to image functional blood flow of microvasculature [29]. Recently, there have been many studies assessing the efficacy of high frequencies for imaging small animals.

Goertz et al. in 2005 were the first to use the nonlinear harmonic signals transmitted at high frequencies (>20 MHz) from microbubbles to differentiate between microbubbles and tissue signals [24]. They further extended their results in use of nonlinear imaging (pulse inversion) of targeted microbubbles with intravascular ultrasound [25]. In 2009, the in vitro experimental studies conducted by Needles et al. proposed a method of using high frequency subharmonic imaging to separate bound targeted microbubbles signals from free bubble signals and tissue [26]. The ultrasound systems used for all the above studies used single-element mechanically scanned transducers which limited contrast enhanced imaging of small animals and also provided
poorer depth of field leading to poor detection of ultrasound contrast agent [27]. A larger depth of field is necessary for better contrast detection, which not only improves the overall image quality but also the consistency of microbubble contrast images [27]. The recent study conducted by Needles et al in 2010 used an array based micro ultrasound system operated in the range of 18-24 MHz for assessing the contrast to tissue ratios obtained from nonlinear fundamental and subharmonic signals in murine kidneys [27]. The largest improvements observed in contrast tissue ratios were for 18 MHz with AM/PIAM and fundamental bandpass filter (13 dB) and for 24 MHz with PI and subharmonic bandpass filter (15 dB).

This thesis work intends to use a similar micro-ultrasound system at a frequency of 24 MHz to depict the tumor microvasculature in murine breast cancer models and obtain and compare the vascularity measures from these images with the ones obtained from a low frequency ultrasound system.
3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Cells and culture material

Human breast adenocarcinoma cells (MDA-MB-231) were purchased from ATTC (Manassas, VA). This cell line shows epithelial-like morphology and has an invasive phenotype in-vitro. In vivo these cells are capable of forming tumors in mammary fat pads of nude rats by intravenous injections.

RPMI-1640 medium (containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate) were purchased from ATCC (Manassas, VA). Penicillin/streptomycin antibiotic was purchased from Sigma Chemical Co., St. Louis, MO. Dulbecco’s Modification of Eagle’s Medium (DMEM) (1X containing 4500mg/L glucose without L-glutamine) was purchased from Mediatech Inc. (Manassas, VA). Trypsin EDTA, fetal bovine serum (FBS), Phosphate buffered saline (PBS) and isopropyl alcohol were all reagent grades and purchased from Fisher Scientific (Springfield, NJ).

3.1.2 Contrast Agent

The contrast agent Definity® (Lantheus Medical Imaging, N Billerica, MA) was used for all the imaging studies. Definity® vial (Perflutren Lipid Microsphere) for injectable suspension consists of lipid-coated microspheres filled with octafluoropropane (C₃F₈) gas. The microspheres contained in Definity® have a mean diameter of 1.1 – 3.3 μm (in vitro measurements) with 98% of the particle population being less than 10 μm in
diameter and a maximum diameter of 20 µm. Definity® is supplied as a single use 2-mL clear glass vial containing clear liquid. Prior to its usage, it is allowed to warm at room temperature and then activated by shaking the vial for 45 seconds using Vialmix®. After activation, 1 mL of Definity® contains approximately $1.2 \times 10^{10}$ Perflutren lipid microspheres [48].

### 3.1.3 Imaging equipment

Two ultrasound scanners, a modified Sonix RP (Ultrasonix, Richmond, BC, Canada) and Vevo 2100 (Visual Sonics, Toronto, ON, Canada) were used for low frequency and high frequency ultrasound imaging respectively.

The Sonix RP scanner provided full access and control of radio frequency (RF) imaging data as well as enabled configuration of new modes like pulse inversion. A L9-4 linear array transmitting at 8 MHz was used in conjunction with this scanner [49].

The Vevo 2100, a micro ultrasound scanner enabled imaging at high frequencies [50]. A MS 250 linear array transducer that provided 30 µm image resolutions through the entire field of view and transmitting at 24 MHz was used for obtaining high frequency scans [50].

A semi-automated histomorphometry system based on a DXC-970 MD color CCD camera (Sony Corporation, Tokyo, Japan) connected to a SMZ-10A microscope (Nikon, Melville, NJ, USA) was used to analyze the pathological tumor specimens using
a motorized stage, controlled by ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA).

3.2 Methods

3.2.1 Cell culturing and injections

The MDA-MB-231 cells were initially thawed to get them to room temperature and then cultured in a medium at 37°C in 5% CO₂. The cells were monitored on a daily basis to keep a check on the medium, the confluence level of the cells, whether they are alive and not contaminated. After they reached 80-85% of confluence, they were sub cultured using 0.25% trypsin (to detach cells adhered to the walls of petri dishes) and growth medium (to stop the enzymatic action of trypsin) and then split into even volumes and incubated at 37°C in 5% CO₂ until they reach a confluence of 75-80%. The sub culturing was repeated until the number of cells was in order of 10⁶. The detailed procedure for cell culturing is provided in Appendix 1.

After culturing, 5 × 10⁶ cells were mixed with matrigel, which helps in forming human tumor xenografts in nude rats. They were injected subcutaneously in right mammary fat pads of 19 athymic immunodeficient rats.

The growth of the tumors was monitored and measured by means of vernier calipers over 3 weeks. Based on the progression of tumor growth, the rats were categorized for scanning studies, conducted after 21, 24 and 28 days of cell injections. The first 8 tumors that grew beyond 5×5×5 mm³ in size were selected for the 21st day of scanning, while the remaining were allowed to grow. On the 23rd day the tumor growth
was again monitored and the 4 tumors that grew more than $5 \times 5 \times 5$ mm$^3$ were selected for 24$^{th}$ day of scanning experiments. Amongst the remaining 7, only the 4 rats that developed tumors were scanned on the 28$^{th}$ day. The remaining three rats that did not show any tumor growth were euthanized. Sixteen out of nineteen rats showed a marked tumor growth with their diameters ranging from 5-13 mm. After the ultrasound scans were obtained, the animals were euthanized by placing them in a gas chamber and saturating the air with CO$_2$. The tumors were surgically removed and scanned to identify the imaging plane that was studied in vivo. All the animal studies were carried out ethically under the supervision of a veterinarian and were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

3.2.2 Acquisition of Ultrasound data

For the imaging studies, rats were intubated and anesthetized with 0.5 to 2 % Isoflurane (Iso-thesis; Abbott Laboratories, Chicago, IL). Also a warming blanket was used to maintain normal body temperature. They were placed in supine position on the operating table.

For imaging method there were 7 intravenous injections in the tail vein of the rats. A saline flush of 0.2cc was given after every injection. For low frequency studies, baseline imaging was done to adjust the output power for optimized pressure measurements and then keep the power constant throughout the studies. This was followed by depth, width and height measurement of the tumor. Transducer was placed so as to image the largest cross-sectional area throughout the studies. Definity was injected (dose: 36µl) via the lateral tail vein of the rats using a 24 gauge needle. Three
contrast injections were administered followed by image acquisition in the right mammary pad of each rat (i.e., over the tumor) with an interval of 3-4 min between the injections, so that the agent had enough time to clear the blood pool. The images were then acquired at the optimal power setting. The final three injections were for scanning the normal tissue (left thigh), which would act as a reference standard and show if there was a clear demarcation between the tumor and normal tissue. High and low frequency scans were done using the respective scanners, as described below.

3.2.2.1 Low frequency SHI

A Sonix RP scanner (Ultrasonix Medical Corp., Richmond, BC, Canada) using a L9-4 linear array transducer (bandwidth: 9-4 MHz) was operated in the Research mode, which enables control of imaging parameters and apply various post-processing and display methods in real-time on RF, I/Q and envelope data to output and store the ultrasound information[50]. The transmit frequency for the transducer was 8 MHz while the echoes were received at 4 MHz (subharmonic frequency). All the parameters used for acquiring the data can be seen in Table 3.1. The Sonix RP scanner was configured to operate in pulse-inversion imaging mode. In pulse-inversion mode, 2 pulses with a 180° phase difference are transmitted and the received echoes are summed, cancelling the first harmonic and other odd linear signals and enhancing the even nonlinear signals. Thus pulse inversion provides an improved contrast to tissue ratio over fundamental imaging. Digital cine clips of LF sub harmonic images were stored for each contrast injection in the hard drive of the scanner.
Table 3.1: Image acquisition parameters in Sonix RP for LF SHI

<table>
<thead>
<tr>
<th>Transducer name</th>
<th>L9-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Pulse Inversion</td>
</tr>
<tr>
<td>Transmit frequency</td>
<td>8 MHz</td>
</tr>
<tr>
<td>Receive Frequency</td>
<td>4 MHz</td>
</tr>
<tr>
<td>Power</td>
<td>-10 dB</td>
</tr>
<tr>
<td>Depth</td>
<td>3 cm</td>
</tr>
<tr>
<td>Pulse repetition frequency</td>
<td>2.56 kHz</td>
</tr>
<tr>
<td>Sampling frequency</td>
<td>40 MHz</td>
</tr>
<tr>
<td>Line density</td>
<td>128</td>
</tr>
</tbody>
</table>

3.2.2.2 High frequency SHI

A Vevo 2100 (Visual Sonics, Toronto, ON, Canada) was used to obtain high frequency scans. MS 250, a linear array micro scan transducer was operated in nonlinear contrast imaging mode transmitting and receiving at 24MHz. Table 3.2 shows the image acquisition parameters used for HF SHI. In nonlinear contrast mode, the amplitude of the ultrasound pulses is modulated so as to utilize the nonlinear response from the microbubbles. Amplitude modulation (AM) sends two ultrasound pulses consecutively with first pulse scaled by a factor relative to second pulse and receives the signal by scaling in response to the second pulse by same factor [42]. It is followed by subtraction of successive echo signals that cancels the linear tissue echoes and retains nonlinear
contrast agent echo signals [42]. Thus it provides better sensitivity by maximizing the ratio of detected contrast agent signals to the residual tissue signals. The digital cine loops of high frequency images were stored in the scanner hard drive for all the injections.

**Table 3.2**: Image acquisition parameters in Vevo 2100 for HF SHI

<table>
<thead>
<tr>
<th>Transducer name</th>
<th>MS 250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Nonlinear contrast</td>
</tr>
<tr>
<td>Transmit frequency</td>
<td>24 MHz</td>
</tr>
<tr>
<td>Receive Frequency</td>
<td>24 MHz</td>
</tr>
<tr>
<td>Power</td>
<td>4%</td>
</tr>
<tr>
<td>Gain</td>
<td>35 dB</td>
</tr>
<tr>
<td>Depth</td>
<td>14 mm</td>
</tr>
<tr>
<td>Width</td>
<td>23 mm</td>
</tr>
<tr>
<td>Dynamic Range</td>
<td>35 dB</td>
</tr>
<tr>
<td>Frame rate</td>
<td>21</td>
</tr>
</tbody>
</table>

**3.2.2.2.1 Filter Design and Implementation**

The high frequency data obtained from Vevo 2100 was acquired with a transmit and receive frequency of 24 MHz. To optimize the RF spectrum of the data for its subharmonic frequency (~12 MHz), the RF data was filtered using various digital filters. Digital filters were designed and tested for their ability to filter the subharmonic signal
component from the ultrasound contrast agent and suppress the background tissue signals.

Individual RF A-lines were extracted from the data frame (332 A-lines with 8960 sampling points per line) with maximum contrast mean power. A subharmonic adaptive algorithm as seen in Appendix 2.a was custom developed in the ultrasound research lab at Thomas Jefferson University, which calculated the fast Fourier transform (FFT) of the RF data and displayed the RF spectrum in Matlab. It was implemented to identify all the signal peaks in the extracted RF A-lines. Figure 3.3 shows a spectrum for one A-line with all the signal peaks marked. The peaks in the vicinity of the sub harmonic components were located from the generated RF spectrum and a band pass filter was developed around this location. This was repeated for all the A-lines in the frame so as to compensate for the variability in the RF signal.
Figure 3.1: A Radio-frequency spectrum generated for one A-line showing fundamental and subharmonic peak.

Twenty bandpass were designed with 4 different orders (starting from 2; the lowest filter order > 0) and 5 different bandwidths estimated to be around the vicinity of subharmonic peak so as to retain the subharmonic component and filter the noise components of the signal. All the filters were assessed qualitatively for image noise and contrast visualization (Table 3.3).
Table 3.3: Classification of 20 Bandpass filters. \( F_s \): sampling frequency. \( F_{c1} \) and \( F_{c2} \): cut off frequencies 1 and 2.

<table>
<thead>
<tr>
<th>Response type</th>
<th>Design method</th>
<th>Order</th>
<th>Frequency Specification (MHz)</th>
<th>Filter no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bandpass</td>
<td></td>
<td></td>
<td>( F_s ) ( F_{c1} ) ( F_{c2} )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IIR Butterworth</td>
<td>2</td>
<td>8 ( F_{c1} ) 16 ( F_{c2} )</td>
<td>BP1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>9 ( F_{c1} ) 15 ( F_{c2} )</td>
<td>BP2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>10 ( F_{c1} ) 14 ( F_{c2} )</td>
<td>BP3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 ( F_{c1} ) 13 ( F_{c2} )</td>
<td>BP4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.5 ( F_{c1} ) 12.5 ( F_{c2} )</td>
<td>BP5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>8 ( F_{c1} ) 16 ( F_{c2} )</td>
<td>BP6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>9 ( F_{c1} ) 15 ( F_{c2} )</td>
<td>BP7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>10 ( F_{c1} ) 14 ( F_{c2} )</td>
<td>BP8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>11 ( F_{c1} ) 13 ( F_{c2} )</td>
<td>BP9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.5 ( F_{c1} ) 12.5 ( F_{c2} )</td>
<td>BP10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>11.5 ( F_{c1} ) 12.5 ( F_{c2} )</td>
<td>BP11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>12 ( F_{c1} ) 14 ( F_{c2} )</td>
<td>BP12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 ( F_{c1} ) 13 ( F_{c2} )</td>
<td>BP13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.5 ( F_{c1} ) 12.5 ( F_{c2} )</td>
<td>BP14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>8 ( F_{c1} ) 16 ( F_{c2} )</td>
<td>BP15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>9 ( F_{c1} ) 15 ( F_{c2} )</td>
<td>BP16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>10 ( F_{c1} ) 14 ( F_{c2} )</td>
<td>BP17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>11 ( F_{c1} ) 13 ( F_{c2} )</td>
<td>BP18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.5 ( F_{c1} ) 12.5 ( F_{c2} )</td>
<td>BP19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>11.5 ( F_{c1} ) 12.5 ( F_{c2} )</td>
<td>BP20</td>
</tr>
</tbody>
</table>

The images selected from an entire cine loop to analyze these filters were the ones at the time point of maximum contrast mean power (figure 3.2). These images were scored by a reader on a visual analogue scale of 1 to 5 (worst to best respectively). The scores of the filters were averaged over each injection and were plotted for each bandpass filter. Figure 3.3 shows an example of scored filtered images.
Figure 3.2: A graph shows wash-in curve fitted to the contrast mean power within the tumor area marked alongside for one contrast injection in nonlinear contrast mode. It provides the data for contrast mean power quantified and smoothed by Vevo software at all the time points in the injection acquired from Vevo 2100.
A Wilcoxon signed rank test was used to compare the maximum scoring filters (on a per rat basis) so as to find out the filter that scored significantly as compared to the others.

The final bandpass filter selected was applied to the data corresponding to the analyzed RF A-line and the filtered image was then generated from that filtered RF data.
This was repeated for all the A-lines in each frame at all the time sequences. The resulting filtered images were then categorized as high frequency subharmonic images (HF SHI).

### 3.2.3 Pathological data

The tumor specimens were placed in 10 % neutral buffered formalin (Fisher Scientific, Houston, TX) for 12-24 hours to fix all the angiogenic markers, dehydrated in graded alcohols, cleaned in xylene and embedded in paraffin using standard methods.

Angiogenesis consists of three stages, activation, migration and proliferation of endothelial cells. The three immunohistochemical markers associated with tumor growth are, vascular endothelial growth factor (VEGF) which promotes tumor growth, invasion and metastasis, cyclooxygenase-2 (COX-2) expressed in response to growth factors, tumor promoters and cytokines, a potent endothelial cell marker platelet endothelial cell adhesion molecule (PECAM or CD31) whose expression is limited to the intercellular junctions of endothelial cells. Each specimen was dissected with slice thickness not more than 4mm and stained immunohistochemically against each of these markers. A monoclonal antibody against PECAM (anti CD31; Dako Corporation, Carpinteria, CA), a polyclonal antibody against COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and a monoclonal antibody against VEGF (Oncogene Research Products, San Diego, CA) were used for staining. Finally, the stained sections were mounted onto glass slides for further analysis.
3.2.4 Image analysis

3.2.4.1 Analysis of pathological data

A semi-automated histomorphometry system based on a DXC-970MD color CCD camera (Sony Corporation, Tokyo, Japan) connected to a SMZ-10A microscope (Nikon, Melville, NJ, USA) was used to analyze the tumor specimens. The histomorphometry system used a 10x objective and a 10x ocular magnification (total magnification 100x) to provide digital images of each histologic slide on the desktop computer. A motorized stage, controlled by ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA) was used to move the specimen until red-green-blue (RGB) images of the entire tumor area were obtained. The blue image which showed tissue enhancement was extracted from the RGB color model. Then the RGB images were converted to hue saturation intensity (HSI) images and the saturation image which indicated vessel enhancement was extracted from HSI color model. The blue image was subtracted from the saturation image and the total area of the tumor was obtained from this image, the tumor was outlined manually on the images where the tumor occupied less than 25% of the entire image area. The subtracted images were enhanced using 3×3 open and sharp filters. The RGB channels were used to segment and count the number of colored or stained pixels as well as total number of pixels in the tumor. Fractional tumor vascularity (FV) was calculated as the number of colored pixels \( c_i \) relative to the total number of pixels \( c_i + x_i \).

\[
FV = \frac{\sum c_i}{\sum c_i + x_i} \tag{1}
\]
The FV measures were obtained for all the specimens stained for each of the immunohistochemical marker (VEGF, CD31 and COX-2) and marker specific pathological maps were created for every tumor specimen using Microsoft Excel 2010. Considering the heterogeneous nature of tumor vasculature, based on the size of each tumor, it was divided into small ROIs of 3-4 mm and the fractional vascularity for each ROI was compared to FV obtained from respective ROIs of ultrasound images (HF SHI and LF SHI).

### 3.2.4.2 Analysis of ultrasound data

The tortuous morphology of tumor angiogenesis was visualized in detail using maximum intensity projection (MIP) technique [51]. MIP technique involves selection of pixels in the image with maximum values, throughout a series of low power grayscale images as the bubbles start replenishing the image plane. An algorithm was developed in the ultrasound research lab of Thomas Jefferson University Hospital to obtain an MIP image of the input image followed by manually outlining the region of interest (ROI) on the MIP image, corresponding to the ROIs marked for tumor specimens. The algorithm calculates the FV by classifying the pixels in the image into tumor vasculature pixels and tumor background pixels and then calculates an optimum threshold value separating these two types of pixels. The algorithm then displayed the composite image showing the tumor vascular architecture for respective region of interest (figure 3.9). This process was performed on high frequency as well as low frequency subharmonic images. All the
algorithms and filters were developed and implemented using MATLAB (R2013a, The Mathworks Inc., Natick, MA).

![MIP image without motion compensation vascular areas](image)

**Figure 3.4**: An example of MIP image of the filtered HF image depicting vascular areas (in red) within a tumor (~7×8 mm) outlined in green.

### 3.2.5 Statistical analysis

The average scores of the bandpass filters used to filter the subharmonic signal from the RF signal of HF SHI were compared using a Wilcoxon’s signed non parametric test.

The data for FV was analyzed on a per ROI basis. Least squares linear regression was used to compare the measures of fractional tumor neovascularity obtained from high
and low frequency subharmonic images with the respective FV obtained from tumor specimens stained for the three immunohistochemical markers for every ROI in all the rat tumors. The correlation coefficients and statistically significant differences (with alpha=0.05) for ultrasound (high and low frequency) and pathological datasets were obtained and compared.

All the statistical analysis was performed using IBM SPSS Statistics 20 (IBM Corporation, Endicott, NY, U.S.)
4. RESULTS

There were $5 \times 10^6$ MDA-MB-231 cells harvested and injected in 19 rats. Sixteen (84%) rats of the 19 developed tumor, while the remaining three did not show any tumor growth and hence were sacrificed before scanning studies. The 16 rats were scanned for LF SHI with 3 contrast injections in the tumor per rat using Sonix RP. However for the HF SHI studies just one successful injection was achieved for 5 of the 16 rats and hence those 5 were discarded. Thus the ultrasound and pathological comparison studies were carried out for the 11 rats each with 3 contrast injections in the tumor (for LF SHI and HF SHI). This study analyzed 33 pathological slides and 66 ultrasound images (including high frequency and low frequency images).

4.1 Results for ultrasound data

All the 11 rat tumors that were scanned had an average diameter of $8.69 \pm 2.23$ mm. Figure 4.1 and 4.2 show the examples of scans for low frequency and high frequency ultrasound imaging, respectively of the same tumor.
Figure 4.1: A scan image obtained from Sonix RP for low frequency at the subharmonic frequency (4MHz) showing the tumor (8×7mm) with its peripheral vascular areas marked.

Figure 4.2: High frequency scan obtained from Vevo 2100 of the same rat tumor as seen in figure 4.1 with marked vascular areas surrounding the tumor.
Both the low and high frequency images of tumor demonstrated the contrast wash-in within the tumor microvasculature. However, the clips obtained from high frequency scans had better resolution throughout the entire field of view and enabled better visualization of tortuous tumor morphology than the low frequency cine loops, which was due to the high transmit and receive frequency.
Figure 4.3: HF image frames depicting the wash-in of the contrast agent injection in nonlinear contrast mode of Vevo 2100 with a B-mode image on the left and contrast image on the right at a.) 2.5 seconds, b.) 4 seconds, c.) 6 seconds and d.) 8 seconds. The tumor area is marked in the B-mode images in a rectangular selection while the contrast images on the right show the arrows marking the contrast enhancement within the tumor as the time of acquisition increases beyond 2.5 seconds.

Figure 4.3 shows the wash-in of contrast agent into the micro vessels of tumor scanned after 28 days of cell injections, in high frequency with Vevo 2100 at different time points. An inflow of contrast microbubbles is seen in the tumor as well as
surrounding vessels within few seconds of contrast injection, depicting intense vascular and less vascular areas within the same tumor and demonstrating the heterogeneous nature of tumor angiogenesis. These images were constructed from RF signal with same receive and transmit frequency (24MHz), hence to extract the subharmonic frequency component from the RF spectrum, the data corresponding to it had to be optimized for subharmonic frequency (~12MHz) to generate respective subharmonic images.

The spectral analysis for high frequency RF data (Figure 3.3) showed a dominant peak around 24 MHz for the transmit frequency and a significant peak at half of this frequency 12 MHz depicting the subharmonic signals from the contrast microbubbles.

![Figure 4.4](image)

*Figure 4.4:* A graph comparing the mean scores for 20 bandpass filters with scores of three maximum scoring filters marked in red arrows
Figure 4.5: A box plot of the mean scores for 20 bandpass filters

Figure 4.4 shows a graph for the score comparison of all the 20 bandpass filters used to filter the subharmonic component from this RF spectrum. Three filters with mean scores BP4: 2.9±0.7, BP9: 3.4±0.8 and BP14: 2.9±0.7 were analyzed further. A Wilcoxon’s signed rank test was performed to compare the mean scores per rat of these three bandpass filters. No significant difference was found between scores of BP4 and BP 14 (p=1.0). The filter BP9’s scores were significantly higher when compared to those of BP4 (p=0.005) and BP14 (p=0.003). Table 4.1 shows the mean score comparison of all the filters. The filter BP9 showed less image noise and provided better contrast visualization as compared to other filters and hence was selected for filtering the subharmonic component from the high frequency image scans in order to create the final SHI images. Table 4.2 provides the frequency specifications for BP9.
Table 4.1: Mean scores and standard deviations of the 20 bandpass filters

<table>
<thead>
<tr>
<th>Filter no.</th>
<th>Mean scores</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP1</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>BP2</td>
<td>2.0</td>
<td>0.1</td>
</tr>
<tr>
<td>BP3</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td>BP4</td>
<td>2.9</td>
<td>0.7</td>
</tr>
<tr>
<td>BP5</td>
<td>1.3</td>
<td>0.7</td>
</tr>
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<td>BP6</td>
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</tr>
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<td>BP7</td>
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<td>BP9</td>
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</tr>
<tr>
<td>BP12</td>
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<tr>
<td>BP13</td>
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<td>0.6</td>
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<tr>
<td>BP14</td>
<td>2.9</td>
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<td>BP15</td>
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<tr>
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<td>BP19</td>
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<td>BP20</td>
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Table 4.2: Design specifications of the bandpass filter BP9 implemented and selected as optimal filter to extract the subharmonic frequency component from the RF spectrum generated by high frequency ultrasound image RF data.

<table>
<thead>
<tr>
<th>Design method</th>
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<tr>
<td>Response type</td>
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<td>Filter order (N)</td>
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</tr>
<tr>
<td>Sampling frequency (Fs)</td>
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<tr>
<td>First cut off frequency (F&lt;sub&gt;c1&lt;/sub&gt;)</td>
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</tr>
<tr>
<td>Second cut off frequency (F&lt;sub&gt;c2&lt;/sub&gt;)</td>
<td>13 MHz</td>
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<tr>
<td>Attenuation at cut off frequencies</td>
<td>-3dB</td>
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</table>

4.2 Pathological data

The corresponding immunohistochemical stains for tumor specimens can be seen in Figure 4.6. Marked staining is seen for areas stained for VEGF and COX-2 in figure 4.6 a and figure 4.6 b. However a diffused staining is seen in case of CD31 expression. Owing to the heterogeneous nature of tumor angiogenesis, the fractional vascularity measures differed significantly within different areas of the same tumor. Hence the tumor neovascularity was decided to be compared on a per ROI basis. Table 4.3 shows a pathological map of the fractional tumor vascularity in corresponding tumor areas obtained from a tumor specimen stained for CD31.
Figure 4.6: Immunohistochemical staining of an area of tumor from Figure 3.1 for (a.) VEGF, (b.) CD31 and (c.) COX-2.
Table 4.3: A tumor specimen was stained for the marker CD31 and mathematical morphometry was performed on the RGB images of the slides using ImagePro software to obtain the fractional vascularity. FV for each tumor area was reported in the pathological map created in Microsoft Excel. Cells marked in yellow, Green and Blue mark the different ROIs in the map.

<p>| | | | | | | | | | |</p>
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</tbody>
</table>
4.3 Comparisons of ultrasound and pathological data

The average fractional vascularity for 11 rats was higher (p<0.05) for low frequency subharmonic imaging (0.38±0.05) as compared to high frequency subharmonic imaging (0.05±0.017). As the frequency of ultrasound increases the signals undergo more attenuation due to which the depth of imaging is limited [47]. Hence LF SHI was able to generate stronger signals which could image the tumor up to 3cm deep and depict more vascular areas, while HF SHI’s imaging depth was limited to 1.4cm. However these
results were not yet correlated with the FV measures obtained from immunohistochemical stains.

Table 4.4 shows the correlation r values and significant p values for each of the comparisons. Linear regression analysis performed on a per ROI basis showed the strongest and significant correlation between tumor vascularity depicted with HF SHI and the percent area stained with VEGF (r = 0.38; p=0.034). The regression analysis also showed a trend towards significance for comparison between HF SHI and CD31 staining (r=0.32; p=0.075) as well as for comparison between LF SHI and COX-2 staining (r=0.31; p=0.08). There was no statistically significant correlation obtained between HF SHI and COX-2 (r=0.018; p=0.08) and for comparisons between LFSHI and VEGF, LF SHI and CD31 (p>0.30). Figure 4.7 shows the scatter plots of FV measures for the ultrasound imaging technique corresponding to the FV measures for respective immunohistochemical markers. Table 4.5 shows comparison the r values and significant p values for comparisons of FVs obtained from HF SHI and FVs of specimens stained for 3 immunohistochemical markers. The only significant values were obtained for expression of VEGF in 21 day rats (r=0.77; p=0.02) and expression of CD31 (r=0.61; p=0.04) in 24 day rats. There were no significant results obtained when the expression of these markers was compared using LF SHI at 3 different time points.
Table 4.4: Comparison results (r values and p values) of linear regression of FVs between ultrasound imaging technique (LF SHI and HF SHI) and three immunohistochemical markers (VEGF, CD31, COX-2).

<table>
<thead>
<tr>
<th></th>
<th>VEGF</th>
<th>CD31</th>
<th>COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF SHI (n=31)</td>
<td>r 0.04</td>
<td>0.18</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>p 0.802</td>
<td>0.316</td>
<td>0.080</td>
</tr>
<tr>
<td>HF SHI (n=31)</td>
<td>r 0.38</td>
<td>0.32</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>p 0.034</td>
<td>0.075</td>
<td>0.464</td>
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</table>

Figure 4.7: a.) Plot of FVs obtained from HF SHI and specimens stained for VEGF against that showed highest significant correlation and b.) and c.) Plots of FV from LF SHI and HF SHI against specimen percent area stained for COX-2 and CD31 respectively. Note that, these plots did not show any significant correlation but showed a trend towards significance.
Table 4.5: Comparison results (r values and p values) of linear regression of FVs between HF SHI and three immunohistochemical markers (VEGF, CD31, COX-2) for 2, 24 and 28 day rats.

<table>
<thead>
<tr>
<th></th>
<th>VEGF</th>
<th>CD 31</th>
<th>COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>HF SHI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 days (n=9)</td>
<td>0.77</td>
<td>0.02</td>
<td>0.34</td>
</tr>
<tr>
<td>24 days (n=11)</td>
<td>0.15</td>
<td>0.67</td>
<td>0.61</td>
</tr>
<tr>
<td>28 days (n=11)</td>
<td>0.56</td>
<td>0.09</td>
<td>0.22</td>
</tr>
</tbody>
</table>
The subharmonic ultrasound images obtained using high frequency ultrasound not only showed better resolution and less image noise, but also depicted improved tumor vasculature as compared to the low frequency images. The only significant correlation found for the measures of FV in our studies was between HF SHI and immunohistochemical staining for the angiogenic marker VEGF ($r = 0.38; p=0.034$; Table 4.5). There was also a trend towards correlation seen in the results obtained for comparisons of HF SHI vs. CD31 and of LF SHI vs. COX-2 ($r\sim0.31; 0.07<p<0.09$; Table 4.5). Thus contrast enhanced subharmonic imaging using high frequency ultrasound appears to provide a non-invasive marker for angiogenesis: particularly for the expression of VEGF.

Earlier studies involving the use of contrast enhanced high frequency subharmonic ultrasound were based on assessing the blood perfusion parameters in small animal models. The study conducted by Goertz et al., showed the subharmonic component of a 20 MHz ultrasound pulse feasible enough to detect the micro vessels in a rabbit ear and mouse heart by differentiating between microbubble and tissue signals [24]. Recent studies conducted by Needles et al using subharmonics of high frequency (>20 MHz) ultrasound pulses to separate bound and free microbubbles from tissue as well as to assess the vascular perfusion in a mouse model using nonlinear contrast imaging techniques showed an improved specificity of microbubble detection [26]. Further studies involving high frequency used nonlinear contrast imaging techniques
(pulse inversion and amplitude modulation) to assess the blood perfusion kinetics in murine kidneys, which provided valuable parameters that help in diagnosing the progression of human disease models [27]. Kodama et al. proposed a method of mapping micro vessels with acoustic liposomes and used high frequency ultrasound to assess angiogenesis and evaluate antitumor effects [52].

There has been plenty of research done and studies conducted in vitro as well as in vivo to assess the tumor vascularization; in particular the process of angiogenesis. Early studies by Schroeder et al assessed the degree of vascularization in 37 malignant melanomas of mice and found an improved correlation between percentage of vessel area and histologically obtained tumor vascularization (p<0.05), but there was no significant correlation between sonographically (using Power Doppler) and histologically obtained grades of tumor vascularization [53]. Iordanescu et al. derived vascularity from contrast enhanced Color Doppler ultrasound images and histological specimens of prostate tumor in 35 genetically engineered mice. The animal models used in this study were genetically altered by linking the prostate-specific rat probasin promoter to the SV40 large T antigen; these models closely mimicked the human prostate cancer progression. [54]. However, they did not obtain any correlations in their results. On the other hand, the set of results obtained by Denis et al. in their experiments on mammary tumor models of 13 rats showed feasibility of Power Doppler sonography in quantitation of tumor angiogenesis. They used the measurement parameters, color pixel density (CPD) and vascularity index (VI). However there were no predictive values obtained for CPD and VI for sensitivity of the tumors to anticancer treatment [55]. Krix et al. achieved significant correlation(p<0.01) between Power Doppler US and histological stains for CD31 marker,
using perfusion parameters such as blood volume, flow and mean velocity in 13 murine tumors [56]. Using a small sample size of 14 mice, significant correlations (p<0.01) were achieved between tumor specimens stained for COX-2 and the vascularity measures obtained from contrast enhanced Power Doppler imaging using human melanoma xenograft tumor models by Forsberg et al. [57]. This study was further extended by increasing the sample size to 28 mice and significant correlations between percent area stained with COX-2 and with VEGF relative to power Doppler and intermittent PI-HI (p<0.05) measures of tumor neovascularity were achieved [58].

There were even more studies conducted to monitor angiogenesis using advanced ultrasound techniques such as microflow imaging, flash echo imaging, contrast pulse sequencing in later half of the decade that showed more positive results. A study by Ro et al. showed significant correlations between vascularity measures obtained from contrast enhanced microflow imaging and percent area stained for COX-2 (p=0.004) and CD31 (p=0.043) and between flash echo imaging and area stained for COX-2 (p=0.026), in a murine glioma models of 21 rats [59]. Similarly, a significant correlation was established between microvessel density count obtained from histological stains of vascular markers expressed by the angiogenic endothelium and the percentage of the circular area enhanced by contrast pulse sequencing ultrasound technique (p < 0.05) in a matrigel model of rats [60]. The contrast enhanced maximum intensity projection of ultrasound images showed significant results for correlation between vascularity from tumor specimens of NMU breast tumor model of rats stained for COX-2 and flash echo images [51]. Furthermore, Forsberg et al. also monitored angiogenesis in human melanoma xenograft models implanted in 30 mice and obtained statistically significant
correlations between vascularity measures obtained from histological staining for the marker VEGF and contrast enhanced ultrasound performed using Power Doppler and pulse inversion harmonic imaging techniques (p<0.01) [58]. In the light of these results, the correlation results obtained in this thesis project between HF SHI and tumor specimens stained for VEGF could also be a significant predictor of angiogenesis in rat tumor models. In 2010, Guibal et al. assessed the effects of anti-VEGF therapy in 32 nude mice. This therapy was able to separate tumors that were rapidly growing from the ones which showed slow growth (p<0.05) by using parameters obtained from contrast enhanced ultrasound examinations and also showed the revascularization of tumors after discontinuing this therapy [61]. A similar study carried out by Ro et al. using contrast enhanced ultrasound monitored the effects of decoy VEGF receptor agent, which blocks VEGF from reaching its natural receptor. They showed positive results (p<0.04) and provided a conclusion that the noninvasive markers for angiogenesis are specific to the tumor cell lines used [62].

The most recent studies in 2013 were conducted by Kang et al. on a rabbit choroidal melanoma model. They evaluated the use of high frequency contrast enhanced ultrasound (30 MHz) and found significant correlation (p<0.04) between the sonographic tumor relative blood volume and histologic tumour vascularity in terms of mean vascular density [63]. Horie et al. detected early stage liver metastasis by monitoring angiogenesis using high frequency contrast enhanced ultrasound (40 MHz) and obtained positive results for correlation (p<0.05) between ultrasound and histological measures of blood vessels density [64]. This indicates that high frequency ultrasound could be a good approach for monitoring angiogenesis noninvasively and owing to the previous work
done for assessing angiogenesis using different ultrasound techniques, it can even act as a valuable marker for endothelial cells of the tumor. This thesis work has proposed the use of high frequency (24 MHz) subharmonic ultrasound to quantify the tumor morphology in rats by means of fractional vascularity which is subsequently seen as a better method than low frequency subharmonic ultrasound imaging in monitoring angiogenesis in rat tumor models.

There are a number of limitations to this study. The number of rats (11) studied was very/quite small, which limits the statistical power of the study and hence the conclusions associated with it. Also a limited number of slides were provided for tumor area and vascularity assessments (3 slides per rat) hence although the entire area of tumor was taken into consideration for the vascularity measurements; the entire lesion vascularity (in 3D) was not evaluated. The elevation thickness of ultrasound beam (mm) is larger than the thickness of specimens by orders of magnitude (µm) due to which an exact match cannot be obtained between ultrasound images and tumor specimens. Few other limitations were; the use of pulse inversion for LF SHI studies as opposed to the use of amplitude modulation for HF SHI which suffers from a reduced signal to noise ratio as compared to pulse inversion, few of the tumors were necrotic in nature and the ultrasound images were also not motion compensated. The xenograft tumor model used cell lines which do not exactly replicate the genetics and histology of human tumors. These cells lack the architectural and cellular complexity of in vivo tumors, which include inflammatory cells, vasculature, and other stromal components [65]. However they also have high degree of predictability and rapidity in tumor formation which makes them suitable for diagnostic studies.
A statistical limitation arises to this study due to the multiple comparisons carried out. Hence a Bonferroni correction may be applied to account for the statistically significant results. This assigns a 0.05 p-value divided by the number of comparisons (here 2 imaging methods×3 immunohistochemical markers= 6) as the p value of significance (Bland and Altman 1995). On the other hand, there are also problems associated with Bonferroni correction. By controlling the group-wise error rate, each individual test is held at unreasonably high standard and makes it likely that legitimately significant results will not be detected [66]. Hence we chose not to adopt the Bonferroni correction for this study.
6. CONCLUSION AND FUTURE WORK

6.1 Conclusion

The overall objective of this thesis was to compare High and Low frequency subharmonic imaging techniques with respect to their ability to depict tumor angiogenesis in a murine breast cancer model.

Both the ultrasound methods successfully demonstrated images of the vasculature in tumors developed in a murine xenograft breast cancer model noninvasively at their respective transmit and receive frequencies, thus fulfilling the first specific aim of this study. The HF SHI (Tx: 24 MHz/Rx ~12 MHz) appears to be a better method than LF SHI (Tx: 8 MHz/ Rx: 4MHz) in monitoring angiogenesis non-invasively. It demonstrated the tumor images with weaker signals but higher resolution than LF SHI.

Three immunohistochemical markers for angiogenesis that were taken into consideration were, Vascular endothelial growth factor (VEGF), Platelet endothelial cell adhesion molecule (PECAM or CD31) and Cyclooxygenase-2 (COX-2). The quantitative parameter, fractional tumor vascularity - derived from contrast enhanced high frequency subharmonic ultrasound images has been shown to provide a noninvasive measure of breast tumor neovascularity in rat xenograft models. The HF SHI images depicted in particular the expression of VEGF in the tumor specimens; albeit based on a limited sample size of 11 rats. The fractional vascularity obtained from HF SHI and LF SHI did not correlate with the FV for expression of CD31 and COX-2 respectively but the results
showed a trend towards significance for their expression. These results would be clearer for an increased sample size.

### 6.2 Future work

The work presented in this study is based on a study conducted on a limited sample size. A large scale study with more rats and other breast cancer cell lines such as NMU mammary gland adenocarcinoma would provide more conclusive results for the expression of all the angiogenic markers in tumor. With an increased sample size, the results obtained for monitoring the expression of all the angiogenic markers (VEGF, CD31 and COX-2) over time by comparing the vascularity measures of rat tumors scanned on different days could be more conclusive.

Tumor angiogenesis is a key factor in the growth of tumor neovascularature and metastasis of the tumor, and hence anti-angiogenic treatment may be an effective way to cease the tumor growth. High frequency subharmonic imaging may become an important noninvasive tool for monitoring antiangiogenic therapies in breast tumor. Based on the conclusion for this study, there could be an expansion of this study further by studying the effects of VEGF inhibitor on the tumor neovascularity of murine xenograft breast cancer models using HF SHI technique.

In conclusion, HF SHI can be seen as a valuable diagnostic tool in monitoring angiogenesis of a breast tumor rat model and can even hold a great potential of monitoring the therapies related to antiangiogenic agents in small animals. These therapies could be further used pre-clinically for assessing breast cancer treatments.
LIST OF REFERENCES


[35] "Entrez Gene: platelet/endothelial cell adhesion molecule".


APPENDIX

Appendix 1: Cell culture protocol

This protocol has been drafted in accordance to the thawing, propagating and cryopreserving protocol of NCI-PBCF-HTB26 (MDA-MB-231) Breast adenocarcinoma [67].

Culture medium preparation:

For a 500 ml bottle of DMEM 1x inject 56 ml of serum (10% of final volume) into the DMEM bottle. Inject 5.6 ml (1% of final volume) of penicillin streptomycin into the DMEM bottle. Swirl it to mix.

Thawing and propagating cells:

• Retrieve a vial of frozen cells.
• Thaw the vial by gentle agitation in a water bath set at 35 °C to 37 °C. To reduce the possibility of contamination, keep the O-ring and cap out of the water.
• Thaw the content with slight shake until only small ice is left in vial. Thawing should be rapid (approximately 2 min to 3 min, just long enough for most of the ice to melt).
• Spray vial with 70% ethanol all over and wipe its surface with clean tissue in the hood.
• Suspend the cells in fresh medium and transfer it to a petri dish.
• Cells are cultured at 37°C in 5% CO₂ and medium is changed about every 3 days.
• It usually takes about 2 days for cells to recovery from freezing. After cell culture reaches 80-85% confluence, subculture is conducted.

**Culturing the cells:**

Observe cells to see how confluent they are, whether the cells are alive, whether the medium hasn’t evaporated and whether the cells are not contaminated. After they reach 80-85% confluence

- Vacuum out all the media from the Petri dishes
- Inject 2-3 ml of PBS into each Petri dish (to wash the cells).
- Vacuum out the PBS of the Petri dishes.
- These cells have a tendency to adhere to the walls of the petri dish while they were harvested. Hence to suspend them we use Trypsin. Injected 2-3 ml of Trypsin into each Petri dish and put the Petri dishes into incubator until the cells detach.
- Use a pipette to measure an even number (e.g. 10 ml) of media and injected all the media measured into the first Petri dish to neutralize the trypsin/cell suspension. Then used the same pipette to remove the floating cells, Trypsin and media from the Petri dish and injected everything into the next Petri dish, repeated until pipette is full or all Petri dishes have been emptied.
- Dispense everything from the pipette into a 50 ml centrifuge tube. Centrifugation makes it easy as to collected the cells from the dispense by separating them from
the rest of the media. Centrifuge (Damon/IEC Division CRU-5000 Centrifuge) –
settings: 4°C, 10 minutes, 1000 rpm, break set to off.

- Vacuum out all the media and Trypsin, leaving a small pellet of cells at the
  bottom of the centrifuge tube.

- Inject (number of Petri dishes x 5 ml – not more than 6 or 7 Petri dishes at a time)
  ml of medium into the tube and redispense/remeasure until no cell pellets are left.
  If there is more than one tube, injected this mix into the next tube as well until no
  cell pellets are left in that tube.

- Divide this mix evenly into an appropriate number of centrifuge tubes (reuse the
  tubes used in previous step) and then add medium to the tubes until the correct
  amount of media/cells are reached (5 ml per Petri dish).

- Put Petri dishes into incubator and monitor the cell density daily. *Incubator
  settings*: 37°C and 5.0% CO₂.
Appendix 2: Matlab functions and subroutines developed for data processing

For reading encoded data from Sonix RP unit, a proprietary function RPead.m was obtained from Ultrasonix Research Forum (http://research.ultrasonix.com). This function loads the encoded data in the Matlab workspace as a variable.

For reading the encoded data from Vevo 2100 unit a propriety C file, VsiExtract.c was obtained from Andrew Needles (Visual Sonics). Running this C file automatically saved the RF data files in csv format and images in jpeg format in the Windows Explorer.

The functions and subroutines described below may be used to process the data further.

**a.) Function: To show A-line spectrum of RF data.**

Input: The RF data files that were obtained from Vevo 2100 for each tumor injection as csv files were loaded in the Matlab Workspace.

Output: The RF spectrum of each A-line (332) for the respective RF data file.

```matlab
function [spectrum_data,FFT_freq] = Alinespectrum(data)
    data = data';
    Fs = 400e6;
    FFT_freq = (0:8960-1)*(Fs/2)/8960;
    fft_plot = [];
    for i = 1:332
        fft_data = 2*abs(fft(data(:,i),17920));
    end
    spectrum_data = fft_data;
    FFT_freq = FFT_freq;
end
```
FFTdata = fft_data(1:end/2);
FFTdata = FFTdata';
FFTdata = smooth(FFTdata);
FFTdata = FFTdata';
figure(1)
plot(FFT_freq/10^6,(FFTdata));
title(strcat('Aline No :',int2str(i)));
xlabel('Frequency (MHz)');
ylabel('Amplitude');
%hold on
if isempty(fft_plot)
    fft_plot = (FFTdata);
else
    fft_plot = [fft_plot;(FFTdata)];
end
%saveas(gcf,['SmoothedAlines_',int2str(i),'_spectrum'],'jpg');
close all
end

fft_plot = mean(fft_plot);
spectrum_data = fft_plot;
figure(2)
plot(FFT_freq/10^6,(fft_plot));
xlabel('Frequency (MHz)');
ylabel('Amplitude');
end
b.) Subroutine: To generate Maximum intensity projection image of an input ultrasound image of the tumor (of both LF SHI and HF SHI) and calculate the vascularity index of marked ROI in an ultrasound image.

Input: An ultrasound image of the tumor in jpeg format

Output: The MIP image of the input image with boundary of ROI (marked in green) and vascular areas (in red) and the value of fractional vascularity for the corresponding ROI in the Matlab command window.

```matlab
%% Clearing Workspace
clear all;
close all;

%% Creating a filelist for accessing images
disp('Creating a file list...')
[filename, pathname] = uigetfile('*.jpg','Select FIRST image file...');

cd(pathname);
files=dir('*.jpg');
filechar = double(filename);

for it = 1:length(filechar)
    if filechar(it) >= 48 & filechar(it) <= 57
        break
    end
end

if it >= length(filechar)
    error('Error ** could not find number in filename.')
end
```
tail = filename(length(filename)-3:length(filename));
numimages = 0;
for ifilenum = 1:200 % Change this 200, if the number of jpegs in the folder exceeds 200
    if exist(filename,'file')
        numimages = numimages + 1;
        filelist{numimages} = filename;
    end
    filename = [filename(1:it-1) num2str(ifilenum) tail];
end
filelist = filelist'; % Output variable contains file list in sequential order

%% MIP and Final image creation

% Reading the first image and converting to grayscale
img11=imread(filelist{1});
img11=im2double(rgb2gray(img11));

% Creating the final image after MIP
[r,c]=size(img11);
final_img=img11;

disp('Creating the final image after MIP...')
for k=1:max(size(filelist))-1
    temp_image=imread(filelist{k+1});
    temp_image=im2double(rgb2gray(temp_image));
    for i=1:r % Implementation of MIP
        for j=1:c
if final_img(i,j) < temp_image(i,j)
    final_img(i,j) = temp_image(i,j);
else
    end
end
end

sprintf('File dealt with: %d', k+1)
end

% Display the final image after MIP and save it
figure(1)
imshow(final_img)
title('MIP image without motion compensation')
%saveas(gcf,strcat('Ver1_MIP_image_without_motion-compensation','.fig'))
%saveas(gcf,strcat('MIP_image_without_motion-compensation','.jpg'))
close all

%%% Selecting the region of the tumor
[x,y,BW,xi,yi] = roipoly(final_img); % The user has to define the polygon

%%% Calculating vascularity index in the tumor
% This is defined as the total number of non-black pixels divided by the
% total number of pixels in the region corresponding to the tumor
tumor_pixels_structure = regionprops(BW, 'PixelList');
% Pixels from the final MIP image corresponding to the tumor
tumor_pixels = tumor_pixels_structure.PixelList;
number_of_tumor_pixels = max(size(tumor_pixels));
% Pixel Values of the original image corresponding to the tumor

for i=1:number_of_tumor_pixels
    row_number=tumor_pixels(i,2);
    col_number=tumor_pixels(i,1);
    pixel_value_tumor_region(i)=final_img(row_number,col_number);
end

% Pixels including vascular component should have higher pixel values
% relative to the tumor background - threshold for tumor background is used

number_of_vascular_pixels=0;
counting_threshold=graythresh(pixel_value_tumor_region);

% Counting the number of pixels corresponding to vascularity

for i=1:number_of_tumor_pixels
    if pixel_value_tumor_region(i) > counting_threshold;
        number_of_vascular_pixels=number_of_vascular_pixels+1;
    else
    end
end

% Calculating and displaying the vascularity index

vascularity_index=number_of_vascular_pixels/number_of_tumor_pixels;
percent_vascularity_index=vascularity_index*100;

sprintf('Vascularity index with threshold %f is : %d',counting_threshold,vascularity_index)

sprintf('Percent Vascularity index with threshold %f is : %d',counting_threshold,percent_vascularity_index)

% To color code and show the identified vascular pixels in the final image
% which were used for vascularity index calculation - visual verification

loc=find(pixel_value_tumor_region>counting_threshold);
vascular_pixel_locations = tumor_pixels(loc,:);
figure(2)

imshow(final_img)
hold on
plot(vascular_pixel_locations(:,1),vascular_pixel_locations(:,2),'.r')
plot(xi,yi,'--g', 'LineWidth',3)
hold off

title('MIP image without motion compensation_vascular_areas')
Appendix 3: List of Statistical tests used

Wilcoxon’s signed rank test:

Wilcoxon’s signed rank test was used to compare the mean scores of images filtered using three Bandpass filters. The filters were implemented on the images acquired at the time point of maximum contrast mean power during an entire injection. All the images were scored on a visual analogue scale of 1 to 5.

Linear Regression Analyses:

Linear Regression was used to compare the fractional vascularities per ROI obtained using two ultrasound methods (HF SHI and LF SHI) and pathological specimens stained for VEGF, CD 31 and COX-2. Each of the comparisons yielded respective r values and significant p values.
Appendix 4: List of software used

1. **Matlab R 2013a** (The Mathworks Inc., Natick WA)

   Purpose: To acquire and process RF ultrasound data. License: Drexel University

2. **IBM SPSS 20** (IBM Corporation, Armonk NY)

   Purpose: For statistical analyses. License: Drexel University

3. **Microsoft Office 2010** (Microsoft Corporation, Redmond, WA)

   Purpose: For record keeping, publishing and presentations. License: Drexel University

4. **Endnote** (Thomas Reuters, New York, NY)

   Purpose: For managing and publishing bibliographies. License: Drexel University