Enhancement of photoacoustic imaging systems for biomedical applications

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SINGAPORE

**ENHANCEMENT OF PHOTOACOUSTIC
IMAGING SYSTEMS FOR BIOMEDICAL
APPLICATIONS**

ARUNIMA SHARMA

SCHOOL OF CHEMICAL & BIOMEDICAL ENGINEERING

2021

ENHANCEMENT OF PHOTOACOUSTIC IMAGING SYSTEMS FOR BIOMEDICAL APPLICATIONS

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A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

2021

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Authorship Attribution Statement

This thesis contains material from 4 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 2 is published as A. Sharma, S. K. Kalva, and M. Pramanik, “A comparative study of continuous versus stop-and-go scanning in circular scanning photoacoustic tomography,” IEEE Journal of Selected Topics in Quantum Electronics 25(1), 7100409 (2019).

The contributions of the co-authors are as follows:

- I wrote the programs needed for comparison, performed mathematical analysis, conducted the experiments required for validation, analyzed the data and prepared the manuscript draft and figures.
- Dr. S. K. Kalva assisted in designing the phantoms and conducting the experiments.
- Assoc. Prof. M. Pramanik proposed the idea of the project, suggested improvements in the set-up, supervised the work, assisted in analyzing the data, and revised the manuscript drafts and figures.

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The contributions of the co-authors are as follows:

- I designed and conducted the experiments required for validation of the idea, analyzed both the experimental and simulation results, and prepared the manuscript draft and figures.
- Ms. Srishti conducted the simulations required for the study.
- Ms. V. Periyasamy designed the programs and supervised Srishti in conducting the simulations.
- Assoc. Prof. M. Pramanik proposed the idea of the project, suggested improvements in the set-up, supervised the work, assisted in analyzing the data, and revised the manuscript drafts and figures.

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The contributions of the co-authors are as follows:

- I proposed the idea of the work and was involved in the initial discussion related to it. I wrote the code for simulation, designed the training algorithm, conducted experiments for validation, analyzed the data, and prepared the manuscript draft and figures.
- Assoc. Prof. M. Pramanik discussed the idea, suggested modifications and improvements, supervised the work, assisted in analyzing the data, and revised the manuscript drafts and figures.

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The contributions of the co-authors are as follows:

- I was involved in initial discussion of the idea of this work. I designed and conducted the experiments required for the study, analyzed the results, and prepared the manuscript draft and figures.
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- Prof. T. Swee-Hin suggested the materials area and supervised the designing of bioengineered eggshell.
- Assoc. Prof. M. Pramanik supervised the project, suggested improvements in the set-up, assisted in analyzing the data, and revised the manuscript drafts and figures.

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Abstract

Photoacoustic imaging (PAI) is a hybrid biomedical imaging modality that combines the advantages of optical illumination and acoustic detection to provide label-free, high resolution images. Among different embodiments of PAI, photoacoustic tomography/ photoacoustic computed tomography (PAT/PACT) is widely used for *in vivo* studies which require deep tissue imaging. Alternatively, photoacoustic microscopy (PAM) is preferred to attain high resolution images at the cost of decreased imaging depth. Due to its advantages like good resolution, high contrast, label-free imaging, deep imaging depth, and the ability to perform multiscale structural and functional imaging, PAI has grown exponentially in the last two decades. However, there still exist some limitations in the current imaging systems in terms of cost, size, acquisitions time, and image quality, that need to be addressed. Therefore, further improvement and optimization of imaging systems is needed so that PAI can be utilized to its highest potential for pre-clinical and clinical applications.

In a typical PAT system, photoacoustic (PA) waves are recorded using an ultrasound transducer rotating around the sample. Among different system configurations, being economical and easily available, single element transducer (SET) based PAT systems are commonly used for biomedical imaging. In these systems, the SET acquires one time resolved PA signal (A-line) for each laser pulse. Cross sectional images are obtained by rotating the SET around the sample and acquiring A-lines at different angles. Acquisition of A-lines can be done either by stop-and-go or by continuous scanning method. By comparing the different types of scanning methods, it was exhibited how the imaging speed of SET-based PAT systems can be improved without compromising the image quality. Mathematical calculations and PA experiments were conducted to show that a 2-4 folds and a 7-12 folds improvement in scan time can be achieved for lasers with low (10 Hz) and high (7 kHz) pulse repetition rate, respectively, while maintaining similar signal to noise ratio, spatial accuracy, and spatial resolution in the images.

Following this, improvement in imaging depth for different PA imaging systems was proposed. Although, PAI provides higher imaging depth compared to optical imaging modalities, depth of PA imaging systems can further be improved by using near-infrared (NIR) waves for illuminating the sample, instead of visible light. By providing lower optical absorption and scattering in biological tissues, and higher maximum permissible exposure (MPE), irradiation by laser pulses in this range can enhance the depth of imaging for PAI. Using Monte Carlo simulations, imaging depth after irradiation in visible, NIR-I, and NIR-II regions were compared. Both PAT and acoustic resolution PAM (AR-PAM) experiments were conducted to validate the simulation results. The results demonstrated that improvement in imaging depth in breast tissue by NIR-II waves is due to the increased MPE in this region.

To further enhance PA images, deep learning (DL) method was suggested to improve the out-of-focus resolution of AR-PAM systems. DL relies on supervised learning, i.e., the network gets trained on pre-defined dataset to solve practical problems on new data. In the last decade, there has been a surge in using DL algorithms for biomedical imaging. A fully dense U-net based Convolutional neural network (CNN) architecture was trained on simulated AR-PAM images and tested on phantom as well as *in vivo* experimental images. Applying the trained model on experimental images showed that the variation in resolution is $\sim 10\%$ across the entire imaging depth (~ 4 mm) in CNN-based images, compared to $\sim 180\%$ variation in original PAM images. The results showed the potential of DL to obtain noise-free, high resolution images.

Versatility of PAI for biomedical applications was explored by using PAT system for chicken embryo imaging. Chicken embryo has been an attractive vertebrate model for biomedical research. Nonetheless, performing label-free imaging of same chicken embryo at multiple developmental stages remains a challenge. We showed the potential of PAT for acquiring high resolution images of live chicken embryos cultured in bioengineered eggshells. Cross-sectional imaging at multiple depths was performed to show the capability of this system for finding the relative depth of different vessels and organs in a growing embryo.

Overall, different features which are essential in defining a biomedical imaging system were compared in this work. We showed methods of improving imaging speed,

depth, and resolution of PAI system and explored application of this modality for chicken embryo imaging.

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Abbreviations

AI	Artificial intelligence
ANSI	American National Standards Institute
AR	Acoustic Resolution
BLI	Bioluminescence imaging
CNN	Convolutional neural network
CT	Computed Tomography
DAS	Delay-and-sum
DAQ	Data acquisition
DL	Deep learning
DMAS	Delay-multiply-and-sum
DS_DMAS	Double-stage delay-multiply-and-sum
DOI	Diffuse optical imaging
EDD	Embryonic day of development
FD	Fully dense
FDA	Food and drug administration
FD-UNet	Fully dense U-Net
FWHM	Full width at half maximum
GPU	Graphics processing unit
HPC	High performance computing center
LDPE	Low density polyethylene
LDU	Laser driving unit
LED	Light emitting diode
MAE	Mean absolute error
MAP	Maximum amplitude projection
MC	Monte Carlo
MCML	MC simulation for light propagation in multilayered tissue
MCEO	MC simulation with embedded object

MPE	Maximum permissible exposure
MRI	Magnetic resonance imaging
MSE	Mean squared error
NA	Numerical aperture
NIR	Near infrared
OAI	Optoacoustic imaging
OCT	Optical coherence tomography
OPO	Optical parametric oscillator
OR	Optical resolution
PA	Photoacoustic
PACT	Photoacoustic computed tomography
PAE	Photoacoustic endoscopy
PAI	Photoacoustic imaging
PAM	Photoacoustic microscopy
PAT	Photoacoustic tomography
PDMS	Polydimethylsiloxane
PET	Positron emission tomography
PLD	Pulsed laser diode
PRR	Pulse repetition rate
PSNR	Peak signal to noise ratio
SAFT	Synthetic aperture focusing technique
SET	Single element transducer
SLN	Sentinel lymph node
SM	Stepper motor
SNR	Signal-to-noise-ratio
SPECT	Single photon emission computed tomography
SSIM	Structural similarity index
US	Ultrasound
USAF	United States Air Force
UST	Ultrasound transducer
UV	Ultraviolet

Chapter 1

Introduction

1.1 Introduction to biomedical imaging

Visualization of internal structures of body has been of great importance for medical practitioners and researchers. It plays a vital role in understanding the anatomy and physiology of body, diagnosing various structural or functional abnormalities, and staging numerous diseases. Advancement in technology has led to continuous evolution of instrumentation and software involved in obtaining biomedical images [1]. Imaging modalities like ultrasound (US), X-ray computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT) are well established and being used by clinicians worldwide [2]. All these modalities help assess the condition of organs or tissues non-invasively. Although these imaging modalities are widely used, there are some limitations associated with them, resulting in constant need of establishment of new imaging systems.

Radiography is one of the oldest imaging modalities which developed after discovery of x-rays in 1895 [3]. In this method, x-rays are projected towards the object and the transmitted rays are detected on the opposite side. Absorption of these

rays is related to the density and structure of the object, thereby resulting in different absorption of radiations by different tissues in body. Thus, when x-rays are projected, the transmitted rays provide a 2D projection of the structure and density of the object in the form of x-ray scans. For x-ray CT, many such 2D projections are taken around the sample and combined to provide a 3D structure [4]. Since there is high absorption of these radiations by most tissues, radiography is widely used for *in vivo* imaging. Similar contrast of two adjoining tissues (e.g., liver/tumor) or contact between soft tissues and bodily fluids can make it challenging to identify the tissue boundary. Nevertheless, use of external contrast agents can also aid in visualizing these tissues as well [5]. Therefore, radiography finds applications in diagnosing fracture, bone diseases etc. Further, screening of lung related disorders like pneumonia, tuberculosis, or lung cancer, are also done using x-ray scans [6]. Moreover, x-ray mammography is widely used for breast cancer screening. Although radiography can provide deep tissue images with good contrast, the body gets exposed to harmful x-ray radiations during the process. High exposure of x-rays can cause tissue trauma and have carcinogenic effects on the body, thus making them unsuitable for long-term imaging.

Ultrasound imaging is another common imaging modality majorly used for examining muscles, tendons, and abdominal organs like pancreas and kidneys [7]. In this method, US waves are sent into the body. These waves strike the tissues and get reflected to varying degrees at tissue boundaries. The degree of reflection depends on the mismatch in impedance of the adjacent tissues. These echoes are detected using ultrasound transducers and images are formed based on the time and strength of received pulses. Thus, the image of internal organs and molecules present in the region of interest can be formed. US imaging is widely used for diagnostic studies in obstetrics, cardiology, oncology, and gastroenterology [8]. It is a cost-effective method which does not use any harmful radiations and provides images of high resolution. However, the images obtained have poor contrast and may have speckle artifacts making the interpretation of images highly operator dependent.

MRI is another imaging modality which does not expose the body to any harmful ionizing radiations and provides high resolution images of internal organs [9]. In this method, the patient is subjected to an oscillating magnetic field which excites the hydrogen atoms (protons) present in water containing tissues of body. This results in generation of radio waves from the protons, which are detected using a receiving coil. Since protons present in different tissues behave differently, their detection can be used to identify the tissue properties and location. Additionally, manipulating the field sequence can help in differentiating fat from water, estimating the blood flow, and imaging structures like bones. Understanding of brain functions is also possible using functional MRI [10]. Since MRI can provide both structural and functional images with high contrast without the use of any harmful radiations or external contrast agents, it is often used for neural imaging, detection of breast tumors, and characterization of lesions in liver [11]. However, for MRI scans, the patients generally have to lie down in a tube-like structure, making it unsuitable for claustrophobic people. Further, use of high magnetic field makes MRI an unsuitable option for imaging people with metallic implants. Additionally, MRI is expensive and has a long scanning time.

Nuclear imaging involves processes wherein a radioactive isotope is administered in the patient either orally or intravenously [12]. PET and SPECT are two common techniques which use nuclear imaging for theranostic purpose. In PET, positron emitting isotopes, known as radiotracers, are attached to an organic molecule (like glucose) and injected in the body, such that they accumulate in the organ of interest. The radiotracers emit positrons which annihilate with electrons inside the body and produce two gamma rays in opposite directions, that can be detected using PET scanners. This determines the line where annihilation occurs [13]. In SPECT, a gamma emitting radioisotope is injected inside the patient. The gamma rays produced by the radioactive isotopes are recorded using gamma cameras at different angles around the body. This gives a 3D tomographic view of internal structures. Both these nuclear imaging techniques used computed tomography reconstruction algorithms to provide cross-sectional images. Compared to PET, the

radioisotopes used in SPECT are generally more easily available making SPECT relatively less expensive. However, the spatial resolution of PET is much better [14]. Nuclear medicine can provide physiological and biochemical information of the body. This method is commonly used to study bone metabolism during healing process, and to visualize distribution of blood flow in muscles. The major disadvantage of these techniques is the incorporation of radioactive substance inside the body, which can have harmful side effects.

Due to the limitations present in the above-mentioned widely used imaging modalities, an improved modality is a need of the hour. To this end, continuous research towards development of non-invasive imaging system has been going on [15]. Since optical imaging techniques give the advantages of safety and good optical contrast, techniques like optical coherence tomography (OCT), and diffuse optical imaging (DOI) gained importance. OCT is an optical imaging modality based on low-coherence interferometry. Near-infrared or visible light is used to illuminate the tissue to be imaged. Detection of back-scattered photons provides information about the scattering properties of underlying biological tissues [16]. High-resolution cross-sectional images depicting various layers of tissues can be formed using this imaging technique [17]. However, since photons scatter in biological medium, it is difficult to get deep tissue images using this method (typical imaging depth is 1-2 mm). Therefore, it is generally used for skin and eye imaging applications. To get images at higher depth, diffuse optical imaging is preferred. DOI offers several centimeters of imaging depth by using diffused photons for illuminations. This technique can therefore image human brain and breast as well [18]. However, compared to OCT, the spatial resolution of images gets compromised. Therefore, achieving high resolution at the depth of a few centimeters is a challenge for purely optical imaging techniques.

The drawbacks of above-mentioned optical imaging technologies have led to rapid evolution of photoacoustic imaging (PAI) [19–27]. PAI [also called optoacoustic imaging (OAI)] is a hybrid imaging modality that combines the advantages of optical excitation and acoustic detection. In this method, the target tissue is

irradiated with nanosecond pulsed laser which leads to generation of acoustic waves around the tissue as a result of transient thermoelastic expansion. Structural and functional information of the tissue can be obtained by acquiring these acoustic waves.

1.2 Photoacoustic imaging - an upcoming imaging modality

PAI is based on the principle of photoacoustic effect, which was discovered by Alexander G. Bell in 1880 [28]. In this effect, absorption of light by any sample causes a transient rise in local temperature, resulting in thermoelastic expansion. This leads to generation of pressure waves, known as photoacoustic (PA) waves. PA waves can be collected using ultrasound detectors and transformed into PA images based on the time of arrival of these waves [29–33]. Being a hybrid imaging modality, PAI offers several advantages: (i) since acoustic scattering in tissues is about three orders of magnitude less than optical scattering, PAI can break through the optical diffusion limit. Therefore, the imaging depth of PAI is higher than purely optical imaging modalities like OCT. (ii) Compared to x-ray, CT, or nuclear imaging modalities, PAI is safer to implement. Usually, light in visible or infrared region is used for irradiating the sample. Unlike x-rays, radiations in these wavelengths are non-ionizing and therefore are considered safer to use. Laser pulses within the safe exposure limit are employed for *in vivo* imaging. (iii) Since different molecules have a unique optical spectrum, it is possible to perform functional imaging using PAI as well. Irradiating the sample at multiple wavelengths can help visualize different molecules simultaneously. For example, dissimilar absorption spectrum of oxy- and deoxy-hemoglobin has been exploited for finding oxygen saturation in blood [34]. (iv) Multiscale imaging is possible with PAI enabling high resolution imaging of biological structures from organelles to organs with same optical contrast. Resolution and imaging depth of PAI are highly scalable such that the ratio of depth to imaging resolution is ~ 200 , making PAI a

Modality Properties	X-ray CT	MRI	Ultrasound	PET	DOT	PAI
Spatial Resolution	~0.5 mm	~0.5-1 mm (3-7 T)	~0.1-0.8 mm (2-15 MHz)	~2-3 mm	~5 mm	~0.1-300 μ m
Good contrast	Hard and soft tissues	Hard and soft tissues	Hard tissues	Contrast agents	Soft tissues	Chromophores (blood, melanin)
Speckle noise	None	None	Strong	None	None	None
Type of radiation	Ionizing	Non-ionizing	Non-ionizing	Ionizing	Non-ionizing	Non-ionizing
Advantages	Good spatial resolution at great depths	Good spatial resolution and good contrast	Good spatial resolution, real time imaging, portable	Functional imaging possible with good contrast	Functional imaging	High intrinsic contrast, Good resolution
Disadvantages	Harmful radiations	High cost, high data acquisition time, not possible on subjects with metal implants	Poor contrast at soft tissue boundaries, Speckle artefacts	Harmful radioactive substances injected, high cost, low resolution	Poor resolution due to strong scattering	Poor resolution at higher depth, bulky system.

TABLE 1.1: Comparison of different imaging modalities

high resolution imaging modality [35]. The application for which PAI is being used defines the depth and resolution needed by the system. Various embodiments of PAI developed to achieve different combinations of depth and resolution have been discussed in subsequent section. (v) Compared to MRI and PET, PAI is faster and less expensive. Multiple applications of real-time PAI have also been demonstrated [36, 37]. Moreover, efforts are being made to further decrease the cost of this imaging system [38, 39]. (vi) Being dependant on optical absorption for generation of signal, this modality mainly provides optical absorption contrast, making it easier to distinguish between different molecules. Non-absorbing background tissue can result in background-free detection of the target molecules. Furthermore, sensitivity of optical absorption is higher in PAI than that in OCT and confocal imaging. (vii) Unlike OCT and US imaging, PAI is free from speckle artifacts. Table 1.1 shows the comparison of PAI with various imaging modalities.

All these advantages have resulted in an exponential growth of PAI in the last two decades. Constant research has led to rapid development of the imaging modality, which is now on its way to clinics for applications in diagnosis of breast cancer [40–42]. PAI has also been explored for multiple structural and functional applications. Imaging of brain [43–45], sentinel lymph nodes (SLN) [46, 47], skin [48, 49], eye [50, 51], ovary [52, 53], thyroid [54], liver [55] urinary bladder [56], bones [57], tumors [58] human extremities [59], intestines [60], and even whole

body small animal [61–63] have been shown using these systems. Functional imaging applications including determining oxygen saturation in blood [64], blood flow speed [65], recording sensory-evoked brain activity [66], monitoring sonothrombolysis [67], quantifying melanin concentration [68], characterizing intestinal structures [69], pH mapping [70], temperature monitoring [71, 72] etc. have also been explored.

1.3 Different embodiments of photoacoustic imaging

Being a multi-scale imaging modality, a range of resolution at different imaging depths can be obtained in PAI by modifying the system configuration. Based on the system configuration, PAI can be broadly classified as photoacoustic computed tomography/ photoacoustic tomography (PACT/PAT), photoacoustic microscopy (PAM), and photoacoustic endoscopy (PAE).

In PAT, the target sample is homogeneously illuminated by the laser. One or more ultrasound transducer(s) detect the emitted PA waves at multiple locations around the sample boundary. Reconstruction algorithms are applied on the acquired PA signals to form cross-sectional image of the target. Volumetric images can be obtained by stacking multiple cross-sectional images. Various types of ultrasound transducer (UST) including single element [73], linear array [74–76], semi-circular array [77], circular array [78], or hemispherical array [79, 80] transducers have been used for detection of PA signal. Majorly two types of configurations – circular PAT and linear array based-PAT - are employed for acquiring PA signals. In circular PAT, PA signal from different angles around the sample is collected and reconstruction algorithms are applied to form cross-sectional image of the target [81]. Volumetric images can be obtained by stacking multiple cross-sectional images. For deep tissue imaging, circular scanning geometry in orthogonal mode is preferred [82]. Such geometry is employed for imaging round objects such as

brain, breast, or entire small animal. Linear array-based PAT is usually used for planar imaging where a linear array transducer is used along with dark-field optical illumination [83]. This configuration is usually used in conjugation with a clinical ultrasound system, for imaging sentinel lymph nodes, urinary bladder etc. Reconstruction algorithms are used to acquire form images from the acquired PA signal [84, 85]. Among various reconstruction algorithms, delay-and-sum (DAS) algorithm is commonly used for both linear and circular PAT. This method adds delay to the PA waves in accordance with the distance between the UST and the sample, and then adds the PA signal accordingly to form the image. Modified DAS algorithms have been developed to improve the tangential resolution in circular PAT [86, 87]. Advancements have also been done for improving reconstruction methods of linear PAT [85]. Delay-multiply-and-sum (DMAS) beamformer was proposed to overcome the limitations of DAS. Further improvement was observed on using DMAS with minimum variance beamforming [88], double-stage DMAS [89], or DMAS with coherence factor [90]. These algorithms have aided in improving the processing time, spatial resolution, and/or SNR of the reconstructed imaged [84]. Although the configuration used for PAT enables deep tissue imaging (up to 4 cm for *in vivo* imaging), the resolution offered by these systems is limited ($\sim 150 \mu\text{m}$). For acquiring images with better lateral resolution, PAM is preferred [91].

PAM employs focused laser excitation or/and acoustic detection to produce high resolution images. Confocal alignment of both the optical and acoustic path may be used to enhance sensitivity of the system. This can be in either reflection [92] or transmission mode [93], depending on whether the light source and the UST are on same side or opposite side of the sample, respectively. Compared to PAT, PAM uses a focused UST with higher central frequency (30-100 MHz) for acquisition of PA waves [94]. By raster scanning the source and the detector in transverse plane, volumetric images are obtained. Using a microelectromechanical systems (MEMS) scanner [95, 96] or a galvanometric mirror [97] can help in decreasing the acquisition time for PAM. Depending on whether the acoustic focus or optical

focus is finer, PAM is categorized into acoustic-resolution PAM (AR-PAM) or optical-resolution PAM (OR-PAM), respectively. OR-PAM utilizes focused light to give optical diffraction limited resolution ($\sim \lambda/2$) in the lateral direction [98–103]. Methods have also been proposed to improve the resolution of OR-PAM beyond this limit [104–107]. However, the penetration depth of OR-PAM is limited by the optical ballistic regime ($\sim 1\text{-}2\text{mm}$), and improvement in resolution generally leads to a corresponding decrease in depth. Increment in penetration depth can be achieved by using AR-PAM but the achievable penetration depth is scalable with the possible imaging resolution. In AR-PAM, a relatively larger area of sample is illuminated, and the UST is focused tightly in the imaging plane [108, 109]. Therefore, the penetration depth of this system can be increased up to optical diffusive limit. Lateral resolution of this system depends on its acoustic focal spot, which in turn varies with the central frequency and numerical aperture of the UST. Increasing the central frequency improves the lateral resolution but also decreases the imaging depth [110]. For both AR- and OR-PAM, the axial resolution depends on the bandwidth of UST. Hence, UST is selected based on the application and the required imaging depth and resolution [29]. To get the advantages of both high resolution and increased imaging depth, integrated AR-and OR-PAM systems have also been developed [111, 112].

For visualizing deeply seated organs at high imaging resolution, PAE can be performed [113, 114]. Miniaturized components for optical illumination and acoustic detection are assembled in an endoscopic probe which is inserted inside the organ to be imaged. Compared to optical endoscopy, higher imaging depth can be achieved using PAE [115]. Therefore, PAE has found various applications for biomedical imaging [116, 117].

Advancements of different systems have led to a surge in biomedical applications of PAI. To further increase the scope of its application, it is also used in combination with other imaging systems like ultrasound imaging [118–121], OCT [122, 123], fluorescence microscopy [50, 124], 2-photon microscopy [125] etc. The increased

interest of clinicians and scientists in PAI substantiate the promising future of this imaging technology for biomedical applications.

1.4 Challenges with current photoacoustic imaging systems

Although PAI has multiple advantages, there are also various challenges associated with the imaging systems that need to be addressed so that the imaging modality can be optimized and employed up to its full potential. Firstly, the imaging set up for PAI is generally bulky and expensive. Secondly, some of the PAI systems have long data acquisition time. Thirdly, intrinsic chromophores or external contrast agents are needed to provide contrast for imaging. Fourthly, although higher than OCT, the imaging depth of PAI is limited by the penetration of light inside the body, and fifthly, it is difficult to get good imaging resolution at deep imaging depths.

To overcome these limitations, multiple steps have constantly been taken. Using laser diode instead of traditional Nd:YAG laser can make the system compact and less expensive, but the laser power of diodes is generally lower, making it difficult to image [126, 127]. Further, to reduce data acquisition time, array transducers can be employed [128]. Circular scan PAT systems use either single element or array based ultrasound transducers for acquisition of generated PA signal. Although, compared to single element transducers, array transducers reduce the acquisition time, they are not very easily available and are usually expensive. Hence, getting a circular scan photoacoustic tomography system which is fast as well as economical remains a challenge.

Depth up to which light can penetrate depends on the tissue to be imaged and on the wavelength irradiating the sample, therefore, selecting the wavelength is crucial. A decreased absorption and scattering coefficient of background tissue may improve the depth of imaging and thus, wavelengths in near infrared region generally provide

higher imaging depth compared to visible region [129, 130]. However, absorption of blood (most common intrinsic chromophore) is relatively smaller in near infrared region, making it difficult to get good contrast. Use of extrinsic contrast agents can help in enhancing the signal thereby waiving the need of internal chromophores [131, 132]. Consequently, more FDA approved contrast agents need to be developed to get high contrast at the irradiating wavelength.

Use of PAM instead of PAT can help in enhancing the resolution, however in PAM the resolution degrades outside the focal plane. System modifications [133, 134] and algorithmic techniques [135, 136] have been used for improving this, but the image quality outside the focal plane is still not at par with resolution at focus. Additional research can help in developing methods that can aid in maintaining good resolution at high imaging depths and outside the focal plane.

To that end, although PAI is one of the fastest growing imaging modalities, there still exist some challenges and limitations that need to be addressed. Hence, the aim of this thesis was to improve and optimize the imaging systems and to broaden the scope of the PAI for preclinical and clinical applications.

1.5 Outline of the thesis

In Chapter 2, we compare different types of scanning methods for single-element transducer (SET) based PAT system. In a circular scanning PAT system, SETs are commonly employed as they offer the advantages of being economical and easily available. Scanning of the SET around the sample can be done using either stop-and-go scanning method or continuous scanning method. Using mathematical calculations and PA experiments, we compare the two scanning methods and analyze the image quality and data acquisition time. We demonstrate that by using continuous scans improvement in data acquisition time can be obtained, without compromising the image quality, signal to noise ratio, or the spatial accuracy of the system. For comparing the two scanning methods, PA images were acquired after

irradiating the sample with either traditional Nd:YAG laser, or with high pulse repetition rate (PRR) pulsed laser diode (PLD). We exhibit that a 2-4 folds and a 7-12 folds improvement in data acquisition time can be accomplished on using lasers with 10 Hz and 7000 Hz PRR, respectively.

In Chapter 3, we present a comparison of photoacoustic imaging depth after irradiation of samples at different wavelengths. First, we use Monte Carlo simulations to show how the fluence in breast tissues changes at different depths for 532 nm, 800 nm, and 1064 nm wavelengths. A corresponding change in absorption of blood and an optical absorber is also shown using these simulations. We then demonstrate the changes in fluence and absorption when the energy density at each wavelength is proportional to the maximum permissible energy at that wavelength. Following this, we validate the simulation results using PAT and AR-PAM experiments. We show that for same energy density, highest penetration depth in breast tissues can be achieved after illumination with 800 nm laser pulses. However, 1064 nm illumination improves the imaging depth due to high permissible energy limit at this wavelength.

In Chapter 4, we present a deep learning based method for improving the out-of-focus resolution of AR-PAM images. We first show simulation of AR-PAM images using k-wave toolbox of MATLAB. A fully dense U-net based deep learning model was trained on these simulated images and tested on experimental AR-PAM images of phantom and *in vivo* rat vasculature. We demonstrate that almost constant resolution across the entire imaging depth (~ 4 mm) can be maintained in CNN-based images, as opposed to $\sim 180\%$ variation in original PAM images. We further validate performance of this method by applying it on *in vivo* rat vasculature imaging. We thereby substantiate that deep learning algorithms can be a promising tool for improving the out-of-focus resolution for AR-PAM systems.

In Chapter 5, we describe application of PAT for chicken embryo imaging. We present a high resolution, label-free method for imaging a live chicken embryo at multiple development stages. Chicken embryos cultured in bioengineered eggshells

were imaged after irradiation by laser pulses at 532 nm and 740 nm. Cross-sectional PA images were obtained for both the wavelengths. We report that biomedical applications of PAT can be extended for imaging chicken embryos. By providing high resolution images of embryonic vasculature with good optical contrast, PAT seems like a promising tool for studying chicken embryo.

Finally in Chapter 6, we summarize this work and discuss about future directions.

Chapter 2

Optimizing scanning methods in circular scanning photoacoustic tomography¹

2.1 Introduction

As has been discussed in Chapter 1, in photoacoustic tomography/photoacoustic computed tomography (PAT/PACT), irradiation of target sample with laser pulses results in generation of PA waves, which are acquired to reveal structural and optical properties of the sample. With each laser pulse, a time resolved PA signal, known as an A-line, is recorded. Various types of transducers can be used for acquiring the generated A-lines. These include - single element transducer (SET), linear array transducer, semicircular array transducer, circular array transducer, or spherical array transducer. Since SET-based systems can collect only one A-line per laser pulse, they have longer data acquisition time (scan-time) than PAT

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systems that use array transducers. Nevertheless, being economical, highly sensitive, and easily available, SETs are still used in PAT/PACT systems [38, 137, 138]. Reducing the scan time for SET-based systems remains a challenge. Use of lasers with high pulse repetition rate (PRR) reduces the scan time. As compared to traditional Nd:YAG laser, pulsed laser diode (PLD) and light emitting diode (LED) offer much higher PRR and therefore, use of these light sources in PAT/PACT system reduces the data acquisition time [126]. Moreover, LED can provide a wide range of wavelengths, making them suitable for spectroscopic analysis. However, due to low pulse energy of laser diodes, signal averaging becomes necessary to acquire images with high signal-to-noise ratio (SNR) [139]. Further research and appropriate system optimization is needed to acquire images with improved SNR and reduced scan time. To further decrease the scan time, one can use continuous scan for data acquisition.

In SET-based systems, acquisition of PA signal can be done in two ways – 1) stop-and-go scan [110], or 2) continuous scan [86]. In former, a motor rotates the ultrasound transducer (UST) around the sample in steps of predefined length. After each step the motor stops and waits for the UST to collect the required number of A-lines, before moving to the next position. This process continues till the UST completes one full rotation (360 degree) around the sample. After this, the A-lines are averaged and later reconstructed to form the cross-sectional image. This contrasts with the continuous scan in which the stepper motor moves the UST continuously around the sample at a constant speed. Multiple A-lines are collected by the moving transducer which stops once it has completed the entire rotation. The A-lines are then averaged, if needed, and reconstructed. Figure 2.1 shows a graphic representation explaining the difference between the two types of scans.

Here, we have compared stop-and-go and continuous scanning methods for SET-based PAT systems for phantoms and *in vivo* imaging of rat brain. We have first shown theoretically that the blurring effects of collecting data from a moving transducer in continuous scans are minimal, whereas there is a noticeable improvement in scan time. For experimental validation, two types of lasers have been used –

FIGURE 2.1: Different types of scanning methods. (a) Stop-and-go scan: transducer stops at various positions (P) to collect the A-line. (b) Continuous scan: a moving transducer collects the A-lines and comes to a stop only after completing full rotation.

traditional pulsed Nd:YAG laser at a PRR of 10 Hz, and a pulsed laser diode at 7 kHz PRR. The experimental results show that the image quality of continuous scans is as good as stop-and-go scans. Further, there is a significant improvement in scan time for continuous scans as compared to the traditional stop-and-go scans.

2.2 Materials and Methods

2.2.1 Nd:YAG-Based PAT Imaging System

The schematic of PAT system using Nd:YAG laser is shown in Figure 2.2(a). A Q-switched Nd:YAG laser (Continuum, Surelite Ex) was used to deliver laser pulses of pulse-width 5 ns at a PRR of 10 Hz and wavelength of either 532 nm or 1064 nm. For phantom (point-source) imaging, a wavelength of 532 nm was used, whereas 1064 nm was used for animal imaging. The laser pulses were reflected towards the sample by using three right angled prisms (PS911, Thorlabs). An optical diffuser was used to homogenize the laser beam. The laser pulse fluence on the surface of sample was maintained at ~ 6 mJ/cm² for 532 nm wavelength, and ~ 25 mJ/cm² for 1064 nm wavelength. Both these values are within the ANSI safety limit, which

FIGURE 2.2: Schematic of the PAT imaging system using (a) Nd:YAG laser and (b) Pulsed Laser Diode. RD: rotating disc, P1–P4: right-angled prisms, UST: ultrasound transducer, SM: stepper motor, PLD: pulsed laser diode, WT: water tank, OD: optical diffuser, LDU: laser driver unit, DAQ: Data acquisition card, R/A/F: receiver/amplifier/filter, TM: Transparent membrane. Photograph of (c) point source phantom, (d) triangle phantom.

is $\sim 20 \text{ mJ/cm}^2$ at 532 nm and $\sim 100 \text{ mJ/cm}^2$ at 1064 nm for a laser with PRR of 10 Hz [140].

For phantom imaging, signal was acquired using a cylindrically focused UST (Olympus NDT, V306-SU-NK, CF = 1.90 in.) of central frequency 2.25 MHz, whereas for animal imaging, an unfocused UST (Olympus NDT, V309-SU) of central frequency 5 MHz was used. Both the transducers had a 13 mm diameter active area and $\sim 70\%$ nominal bandwidth. A stepper motor (Lin Engineering, Silverpak 23C) was used to rotate the UST around the sample. The UST was placed in a water tank to enable better coupling of the ultrasound signal. Once acquired, the PA signal was amplified by $\sim 45 \text{ dB}$ and filtered by a pulse receiver (Olympus-NDT,

5072PR) and then recorded using a data acquisition card (DAQ) (GaGe, compuscope 4227) inside a desktop (Intel xeon 3.7 GHz 64-bit processor, 16 GB RAM). All the data was collected at a sampling rate of 25 MS/s. A sync signal from the laser was used to synchronize the data acquisition and laser excitation.

2.2.2 PLD-Based PAT Imaging System

The schematic of PLD-PAT system is shown in Figure 2.2(b). Laser pulses at ~ 803 nm wavelength and 136 ns pulse width were provided by the pulsed laser diode (Quantel, France). A laser driver unit (LDU) was used to manipulate the laser pulse. This LDU consisted of a temperature controller (LaridTech, MTTC1410), a 12 V power supply (Votcraft, PPS-11810), a variable power supply (BASETech, BT-153), and a function generator (FG250D, Funktions generator). The PLD generated ~ 1.4 mJ of energy (at a PRR of 7 kHz) per pulse, which passed through an optical diffuser (for uniform illumination) to illuminate ~ 16 cm² on the sample surface. Laser fluence on sample surface was 0.05 mJ/cm². For a wavelength of 803 nm, the maximum permissible exposure (MPE) for an exposure of over 10 seconds is ~ 317 mW/cm² [140]. Thus, for a PRR of 7000 Hz, MPE is ~ 0.045 mJ/cm². For comparison of the two types of scans, the scan-time for our experiments was more than 10 seconds, and therefore the laser fluence exceeded MPE in some cases marginally. However, it has been shown previously that image acquisition using PLD-PAT system can be done within 5 seconds, without any safety limit violation [44]. The sync signal from the function generator synchronized the data acquisition and laser excitation. PA signal was acquired using a flat 2.25 MHz UST (Olympus NDT, V306-SU). The characteristics of UST, stepper motor, as well as DAQ were the same as those used for Nd:YAG based PAT system. Once collected, the A-lines were averaged and later delay-and-sum algorithm [86] was employed to reconstruct the cross-sectional PAT images.

2.2.3 Data Acquisition

Continuous scans are generally faster than stop-and-go scans. If the angular velocity (ω) and the angular acceleration (α) are known for a motor which completes its rotation in N steps, time taken for it to move around the sample is given by

$$t_{rotation} = \frac{2\pi}{\omega} + \frac{N\omega}{\alpha}. \quad (2.1)$$

For continuous scans, $N = 1$ and no extra time is required for collection of A-lines. Therefore,

$$t_{continuous} = \frac{2\pi}{\omega} + \frac{\omega}{\alpha}. \quad (2.2)$$

If the same number of A-lines is to be collected for both types of scan method, total time taken for stop-and-go scan will be

$$t_{stop_and_go} = t_{continuous} + t_{rotation}. \quad (2.3)$$

For continuous scans, the time of rotation depends only upon the number of A-lines to be collected, whereas for stop-and-go scans, time can be varied by varying the number of steps, velocity, and acceleration of the motor, while keeping the number of A-lines collected constant. The time of rotation can be decreased by decreasing the number of steps, but that increases the angle between two consecutive positions where A-lines are collected, thereby deteriorating the image quality. Another method to decrease the scan time is by increasing the velocity and acceleration of the motor. However, this may result in increasing vibrations in the motor and therefore, is limited.

Although continuous scans are faster than stop-and-go scans, the latter are still preferred in some cases. For example, the blurring caused in continuous scans due to collection of A-lines by a fast-moving UST may be a deciding factor to choose stop- and-go scan. Nonetheless, it can be shown mathematically that the blur is inconsequential as compared to the resolution of PAT systems. If the sample

diameter is d mm, and speed of sound in water $1.5 \text{ mm}/\mu\text{s}$, time taken for acoustic waves from the entire sample to reach the UST will be

$$t = \frac{d}{1.5} \mu\text{s}. \quad (2.4)$$

If the transducer completes one rotation in T seconds, angular displacement of the transducer while it collects acoustic waves from the furthest point of the sample is given by

$$\theta(\text{radian}) = \frac{2\pi}{T} \times \frac{d}{1.5} \times 10^{-6}. \quad (2.5)$$

During this time, the maximal displacement of sample with respect to transducer will be approximately,

$$\approx \frac{2\pi}{T} \times \frac{d}{1.5} \times 10^{-6} \times \frac{d}{2} \text{mm}. \quad (2.6)$$

Typically, the sample diameter is $\sim 15 \text{ mm}$ (say, small animal brain imaging), and the fastest scan time that has been reported so far is 3 seconds for completing one rotation [126]. Computing (2.6) using these values shows that the maximum displacement of the sample with respect to the transducer before it can record PA signal from across the sample, is $\sim 0.15 \mu\text{m}$, which is insignificant compared to the resolution of the PAT system ($\sim 182 \mu\text{m}$ with 5 MHz UST, and $\sim 381 \mu\text{m}$ using 2.25 MHz UST [126]).

However, it should be noted that the pulse energy of nanosecond lasers fluctuates by about 20% between individual pulses. In stop-and-go scans, the fluctuation can be compensated for, by collecting multiple A-lines at the same point and averaging them. Moreover, averaging also helps in improving the SNR, which in turn improves the image quality. Averaging of signal for continuous scans is also possible, but motion of the UST results in relative motion of sample between two A-lines [141]. This relative distance depends on the speed of the UST and the PRR of the laser.

For a UST, the relative displacement of sample between 2 A-lines, when the PRR of laser is rep Hz, will be

$$\approx \frac{2\pi}{T \times rep} \times \frac{d}{2}. \quad (2.7)$$

If we consider a PAT system wherein a sample of diameter 15 mm is imaged in 18 seconds, the distance moved by transducer between two A-lines for a 7 kHz laser will be 0.3 μm . If M A-lines are averaged together, the distance moved by the transducer becomes M fold. Therefore, for this system, if 500 A-lines are averaged, the relative displacement becomes 150 μm . Averaging is common while using lasers with high PRR like PLD and LED. The pulse energy of these lasers is usually low, which results in a low SNR of the image. Averaging is done to improve the SNR. Since the relative displacement of UST is inversely proportional to the PRR of laser, blurring caused due to this averaging is generally negligible. Nevertheless, blurring can result in a decline in image quality and therefore it should be taken care that the relative displacement remains within the limits of resolution even after averaging. For fair comparison, here, the averaging done for continuous scans is same as that done for stop-and-go scans.

In case of systems imaging larger objects, for example, breast imaging system, the sample diameter can be up to 15–20 cm [79, 142, 143]. Hence, there is an increase in both the relative displacement of UST while collecting signal from the sample, as well as the relative displacement of sample between collections of two consecutive A-lines. The effect of blurring due to continuous scan might be more prominent there. Thus, evaluation of speed at which the UST can rotate and the signal averaging that can be done becomes crucial and should be performed before choosing the optimum parameters for such imaging systems.

Another factor to be kept into account while doing a continuous scan is the Doppler effect. Due to relative motion of the sample and the UST, there is a shift in the detected ultrasound signal frequency. The frequency shift for an object moving at a velocity v relative to the detector (UST) at an angle θ is given by

$$\frac{\Delta f}{f} = \frac{v \cos \theta}{c} \approx \frac{2\pi}{T} \times \frac{d}{2} \times \frac{\cos \theta}{c} \quad (2.8)$$

where, c is the speed of acoustic waves in medium (water). If we again consider the typical values of $d = 15$ mm, $T = 3$ seconds and $c = 1.5$ mm/ μ s, the maximum Doppler frequency shift would be $\sim 0.001\%$ (50 Hz for 5 MHz frequency component), and therefore is inconsequential and can be ignored [141].

2.2.4 Phantom Imaging

Two phantoms were used to compare the two scanning methods. A point source phantom was imaged using Nd:YAG based PAT system and a shape phantom was imaged using PLD-PAT system. The point source phantom was prepared using five pencil leads of diameter 0.5 mm. These leads were stuck inside pipette tubes which were fixed vertically on an acrylic slab. The pipette tubes were used to keep the leads stable and vertical [see Figure 2.2(c)]. One lead was placed at the scanning center and the other four were placed at ~ 1 cm distance in four different directions from the center lead. For comparison, 180, 360, 720, 1440, and 2880 A-lines were collected for both the types of scans. For stop-and-go scan, the step size was kept 1° , 2° , and 4° in different scans, and then the desired total number of A-lines was collected by changing the number of A-lines collected after each stop. For example, when total number of A-lines collected was 720, the UST collected 2 A-lines per step when step size was 1° , 4 A-lines per step when step size was 2° , and 8 A-lines per step when step size was 4° . To change the number of A-lines collected for continuous scan, the speed of the motor was changed. Since the PRR of laser was fixed at 10 Hz, collection of 180, 360, 720, 1440, and 2880 A-lines was done by rotating motor such that it completed one rotation in 18, 36, 72, 144, and 288 seconds, respectively. Before reconstruction, averaging of A-lines was done. For stop-and-go scans, averaging was in accordance with the step size of motor, such that, finally 360, 180, and 90 A-lines were reconstructed, respectively.

For fair comparison, number of A-lines reconstructed for continuous scans was 360, 180, and 90 as well.

Another phantom used for the experiments was a triangle made of horsehair of $\sim 120\text{ }\mu\text{m}$ diameter. Three hairs of length $\sim 1\text{ cm}$ were stuck with each other to form a triangle. This triangle was held using three pipette tips fastened on an acrylic slab. Figure 2.2(d) shows a photograph of the triangle phantom. Images of triangle phantom were taken using PLD-PAT system. A-lines were collected by moving the UST around the sample in steps of 1° , 2° , and 4° (stop-and-go scans) and continuously for 9 and 18 seconds (continuous scans). Number of A-lines collected in 9 and 18 seconds were 63000 and 126000, respectively. Same numbers of A-lines were collected for different step sizes of stop-and-go scans as well. Averaging was done similar to the Nd:YAG-PAT system.

2.2.5 *In vivo* Imaging

Healthy female rats weighing $\sim 100\text{ g}$ were procured from InVivos Pte. Ltd., Singapore and were used for conducting the *in vivo* imaging. Experiments were conducted in conformity with the approved guidelines and regulations by the institutional Animal Care and Use committee of Nanyang Technological University, Singapore (Animal Protocol Number ARF- SBS/NIE-A0263). The rats were anaesthetized using a combination of Ketamine (120 mg/kg) and Xylazine (16 mg/kg) injected intraperitoneally (dosage of 0.2 ml/100 g). Hair on the head of rat was epilated using hair removal lotion. A mixture of 1.0 L/min oxygen and 0.75% isoflurane was used to keep the rat anaesthetized during the experiment. The rat, along with its holder, was mounted on a translational stage which was used to adjust its height. The water tank was designed such that it had a hole in the center which was large enough to accommodate the rat's head. This hole was sealed using a clear, flexible membrane. The height of rat was adjusted such that its head protruded through the hole and was aligned in the same horizontal plane as the

transducer. After scanning, the rat was euthanized by intraperitoneal injection of Valabarb (sodium pentobarbitone: 300 mg/ml).

2.3 Results and Discussion

2.3.1 Image Quality

The reconstructed images of point source phantom are shown in Figure 2.3(a)–(r). Reconstructed images obtained on collecting 360 A-lines, 720 A-lines, and 1440 A-lines are shown in Figure 2.3(a)–(f), (g)–(l), and (m)–(r), respectively. The reconstructed images of A-lines collected by employing stop-and-go scanning method are shown in Figure 2.3(a)–(c), (g)–(i), and (m)–(o) and those collected by using continuous scans are shown in Figure 2.3(d)–(f), (j)–(l), and (p)–(r). For both the types of scans, images formed on reconstruction of 90 [see Figure 2.3(a), (d), (g), (j), (m), and (p)], 180 [see Figure 2.3(b), (e), (h), (k), (n), and (q)], and 360 [see Figure 2.3(c), (f), (i), (l), (o), and (r)] A-lines are shown. It can be observed from the images that there is an improvement in image quality with an increase in number of steps (reconstructed A-lines), as expected. However, there is no difference in terms of visual image quality for both types of scanning methods.

Figure 2.4(a)–(l) are the images of triangle phantom acquired after PLD-PAT scans. Figure 2.4(a)–(f) and (g)–(l) were images obtained when 63000 A-lines and 126000 A-lines were collected, respectively. Before reconstruction, the A-lines were averaged according to the steps of stop-and-go scans. 90 A-lines were reconstructed in Figure 2.4(a), (d), (g), and (j), 180 in Figure 2.4(b), (e), (h), and (k), and 360 in Figure 2.4(c), (f), (i), and (l). It was observed in these images that although the shape of phantom was retained, some artefacts were present in the background when the number of A-lines reconstructed was less. A noticeable decrease in artefacts was observed when the number of A-lines reconstructed increased from 90 to 180 and further to 360.

FIGURE 2.3: Comparison of PAT images of point source phantom using (a-c, g-h, m-o) stop-and-go and (d-f, j-k, p-r) continuous scanning methods. (a-f) 360, (g-l) 720, and (m-r) 1440 A-lines were collected by moving the motor in (a, g, m) 90, (b, h, n) 180, and (c, i, o) 360 steps. The images obtained using continuous scans were averaged in (d, j, p) 90, (e, k, q) 180, and (f, l, r) 360 A-lines. Scale bar as shown in (a).

FIGURE 2.4: PAT images for triangle sample for comparison of (a-c, g-i) stop-and-go and (d-f, j-l) continuous scanning methods. (a-f) 63000 and (g-l) 126000 A-lines were collected by scanning continuously for (d-f) 9 sec and (j-l) 18 sec and by scanning in (a, g) 90, (b, h) 180 and (c, i) 360 steps. The image was reconstructed by averaging the acquired A-lines in (a, d, g, j) 90, (b, e, h, k) 180, and (c, f, i, l) 360 A-lines. Scale bar as shown in (a)

For both the phantoms, SNR was used to quantify the image quality. To compute SNR, the peak to peak amplitude of PA signal (V) was divided by the standard deviation of the background noise (n). Thus,

$$SNR = \frac{V}{n} \quad (2.9)$$

A small region around the corner of the image, where there was no PA signal, was

Laser(PRR)	A-lines collected	90 A-lines reconstructed		180 A-lines reconstructed		360 A-lines reconstructed	
		Stop-and-go	Continuous	Stop-and-go	Continuous	Stop-and-go	Continuous
Nd:YAG (10 Hz)	180	23.9	23.4	24.3	24.1	N/A	N/A
	360	24.9	24.4	25.1	24.9	27.2	26.6
	720	25.5	26.4	27.6	27.7	29.2	30.5
	1440	26.2	26.6	29.3	28.7	33.2	33.3
	2880	27.6	26.9	28.8	28.3	36.3	36.1
PLD (7 kHz)	63000	16.5	17.7	25.8	26.7	34.5	34.4
	126000	16.6	17.2	24.1	25.3	35.5	35.6

TABLE 2.1: SNR (dB) for phantom imaging

considered the background and n was computed using that area. V was calculated by taking an average of all the PA signals above a constant threshold value, in the area of interest. Table 2.1 shows the SNR of the different scans of point source phantom and triangle phantom. Several observations can be made from the table. Firstly, SNR increases with an increase in number of A-lines collected, provided the number of A-lines reconstructed is kept constant. This effect was up to 8% when the number of A-lines collected by Nd:YAG laser was doubled. However, no significant difference in SNR was observed in images obtained using PLD. Secondly, for a fixed number of A-lines collected, SNR value improved between 8% and 50% when the number of A-lines reconstructed increased from 90 to 360. Thirdly, if the number of A-lines collected and the number of A-lines reconstructed are kept the same, the SNR changed between 2–5% for stop-and-go scans and continuous scans.

For further comparison, the images obtained from different scans of point source phantom were evaluated for spatial accuracy. This was done by calculating the average distance of the center pencil lead from the other four leads. It was observed that the distance remained between 9.33 mm and 9.4 mm irrespective of the type of scan, number of A-lines collected, or the number of A-lines reconstructed. Repeating the experiments resulted in a change in distance of less than 0.6%. Therefore, it can be concluded that continuous scan does not affect the spatial accuracy of the system.

Following this, *in vivo* experiments were conducted using both Nd:YAG-PAT system as well as PLD-PAT system. The PAT images obtained are shown in Figure 2.5 (using Nd:YAG laser), and in Figure 2.6 (using PLD). While using Nd:YAG

FIGURE 2.5: Comparison of *in vivo* brain PAT images of female rat for (a–c, g–i, m–o) stop-and-go and (d–f, j–l, p–r) continuous scanning methods. (a–f) 360, (g–i) 720, and (m–r) 1440 A-lines were collected and were averaged in (a, d, g, j, m, p) 90, (b, e, h, k, n, q) 180, and (c, f, i, l, o, r) 360 A-lines corresponding to the number of steps taken in stop-and-go scans. Scale bar is shown in (a). Photographs of rat brain (s) before and (t) after skin removal are shown.

laser, 360, 720, and 1440 A-lines were collected. For continuous scans, this was achieved by moving the stepper motor at a velocity of $10^\circ/\text{second}$, $5^\circ/\text{second}$, and $2.5^\circ/\text{second}$, respectively. For stop-and-go scans, the velocity of motor was optimized at $\sim 7^\circ/\text{second}$. Figure 2.5(a)–(f), (g)–(l), and (m)–(r) show images obtained when 360, 720, and 1440 A-lines were collected, respectively. Among these, Figure 2.5(a)–(c), (g)–(i), and (m)–(o) were acquired using stop-and-go scans whereas Figure 2.5(d)–(f), (j)–(l), and (p)–(r) were acquired using continuous scans. Corresponding to the number of steps of stop-and-go scans, the A-lines of both stop-and-go and continuous scans were averaged to 90 A-lines [see Figure 2.5(a), (d), (g), (j), (m), and (p)], 180 A-lines [see Figure 2.5(b), (e), (h), (k), (n), and (q)], and 360 A-lines [see Figure 2.5(c), (f), (i), (l), (o), and (r)]. Photographs of the rat before and after skin removal are shown in Figure 2.5(s) and (t), respectively.

SNR values for *in vivo* images are shown in Table 2.2. An improvement in image

quality and SNR was observed on increasing the number of collected A-lines, for both the types of scans. When the number of A-lines collected increased from 360 to 1440, SNR increased by $\sim 27\%$. A similar increase in SNR was observed when the number of A-lines reconstructed was increased from 90 to 360. Visually, the images appeared smoother and had lesser artefacts when more number of A-lines was collected and/or reconstructed. However, a maximum of just 7% change in SNR was observed between the images for stop-and-go scans and continuous scans when number of A-lines collected and reconstructed were kept constant. Moreover, there were instances when SNR of continuous scans was better than stop-and-go scans. Therefore, such changes in SNR values can be considered negligible.

FIGURE 2.6: Experimental results of *in vivo* brain images of female rat using PLD-PAT system. Images were acquired using (a–c) stop-and-go and (d–f) continuous scanning methods. 126000 A-lines were collected by moving the motor in (a) 90 steps, (b) 180 steps, (c) 360 steps, and (d–f) continuously for 18 sec. The collected A-lines were averaged into (a, d) 90 A-lines, (b, e) 180 A-lines, and (c, f) 360 A lines before reconstruction. Scale bar is same for all images as shown in (a). Photographs of brain are shown (g) before and (h) after skin removal.

For rat brain imaged using PLD-PAT system, velocity of motor for continuous scans was fixed at $20^\circ/\text{second}$, i.e., it completed one rotation in 18 seconds collecting 126000 A-lines. For stop-and-go scans, the motor completed one rotation in 90, 180, and 360 steps with a maximum velocity of $\sim 7^\circ/\text{second}$ between two stops. 126000 A-lines were collected by collecting 1400, 700, and 350 A-lines per step,

Laser(PRR)	A-lines collected	90 A-lines reconstructed		180 A-lines reconstructed		360 A-lines reconstructed	
		Stop-and-go	Continuous	Stop-and-go	Continuous	Stop-and-go	Continuous
Nd:YAG (10 Hz)	360	15.9	15.9	18.8	17.8	19.2	18.2
	720	17.6	17.2	21.2	20.4	21.8	20.5
	1440	18.2	18.6	24.2	22.6	24.5	23.3
PLD (7 kHz)	126000	17.8	17.1	21.4	20.6	22.2	20.7

TABLE 2.2: SNR (dB) for *in vivo* rat brain imaging

respectively. Images acquired by moving UST in steps of 1° , 2° , and 4° (stop-and-go scans) are shown in Figure 2.6(a)–(c), respectively. Figure 2.6(d)–(f) were acquired by collecting A-lines continuously, but finally averaging them into 90, 180, and 360 A-lines, respectively, before reconstruction. Photographs of rat taken before [see Figure 2.6(g)] and after [see Figure 2.6 (h)] skin removal are shown to verify the acquired images.

SNR of these images are also given in Table 2.2. When the number of A-lines reconstructed was increased from 90 to 360, a $\sim 27\%$ increase in SNR was observed for stop-and-go scan and $\sim 21\%$ increase for continuous scan. However, for the same number of A-lines reconstructed, $\sim 7\%$ decrease in SNR was observed in continuous scans as compared to stop-and-go scans. For most cases, this decrease is not prominent and therefore can be considered inconsequential. Thus, the results obtained were similar to those obtained using Nd:YAG based PAT system. The increase in speed of PLD based system was compensated by the increase in number of A-lines collected due to higher PRR. Therefore, although the transducer was moving at a higher speed, the blur due to continuous scans was insignificant.

Another parameter to compare image quality is resolution of the system. Images of triangle phantom were used for the same because the diameter of horsehair used was less than the expected resolution of the system. Line profiles along different cross sections of horsehair (i.e., perpendicular to edges of triangle) were plotted and fitted to a Gaussian curve. Full width at half maximum values of this curve provided the resolution.

Resolution along different line profiles were averaged for more accurate measurements. Table 2.3 shows the calculated resolution for different types of scans. An

A-lines collected	90 A-lines reconstructed		180 A-lines reconstructed		360 A-lines reconstructed	
	Scan1	Scan2	Scan1	Scan2	Scan1	Scan2
63000	343	363	348	357	345	355
126000	339	360	339	353	352	351

TABLE 2.3: Resolution (μm) for different scans of PLD-PAT system. Scan1: Stop-and-Go Scan, Scan2: Continuous Scan.

Laser	Stop-and-go (90 steps)		Stop-and-go (180 steps)		Stop-and-go (360 steps)		Continuous Scan	
	Scan time	Laser pulses	Scan time	Laser pulses	Scan time	Laser pulses	Scan time	Laser pulses
	(A-lines)		(A-lines)		(A-lines)		(A-lines)	
Nd:YAG (10 Hz)	80s (180)	800	95s (180)	950	N/A	N/A	18s (180)	180
	98s (360)	980	108s (360)	10800	109s (360)	1090	36s (360)	360
	135s (720)	1350	147s (720)	1470	145s (720)	1450	72s (720)	720
	210s (1440)	2100	217s (1440)	2170	217s (1440)	2170	144s (1440)	1440
	355s (2880)	3550	362s (2880)	3620	364s (2880)	3640	288s (2880)	2880
PLD (7 kHz)	78s (63000)	546000	90s (63000)	630000	125s (63000)	875000	9s (63000)	63000
	87s (126000)	609000	99s (126000)	693000	132s (126000)	924000	18s (126000)	126000

TABLE 2.4: Comparison of scan time and laser exposure (number of pulses the sample got exposed to) for continuous and stop-and-go scans

improvement in resolution of stop-and-go scans as compared to continuous scans, can be observed in the table. The improvement is more prominent (4–6%) when 90 A-lines are reconstructed and negligible (1–3%) when 360 A-lines are reconstructed. Furthermore, for continuous scans, the resolution improves when more number of A-lines is reconstructed. No such trend is however visible in stop-and-go scans.

2.3.2 Scan time and Laser Exposure

As shown above, scan time for stop-and-go scans will be much higher than continuous scans if maximum speed and acceleration are kept constant. For continuous scans, the maximum speed is related to the number of A-lines collected and therefore the speed cannot be manipulated. This is not the case with stop-and-go scans, where the speed is limited by the motor and the vibrations it can cause and not by the number of A-lines. Before performing the experiments, the velocity and

acceleration of stepper motor were optimized such that scan time was the least and vibrations minimal.

An increase in scan time not only prevents real-time imaging, but also results in higher laser exposure. In continuous scan, the sample is exposed to laser only during imaging, when it is necessary. In contrast, in stop-and-go scans, apart from imaging time, the sample is exposed to laser while the transducer is moving around the sample. This is a concern especially for biomedical imaging. Table 2.4 shows the scan time and the number of laser pulses the sample got exposed to. From Table 2.4 it can be observed that continuous scans were ~ 4 times faster than stop-and-go scans when lesser number of A-lines (180 and 360) were collected and ~ 1.5 times faster when higher number of A-lines were collected, for Nd:YAG-PAT system. Further, decrease in acquisition time for continuous scans as compared to stop-and-go scans was 7–12 folds for PLD-PAT system, when same number of A-lines were acquired in both. This resulted in a significant decrease in laser exposure of the sample particularly for PLD-PAT.

Another observation made from Table 2.4 was that for the same number of A-lines collected, the data acquisition time increased by only $\sim 10\%$ when the number of steps was increased, in case of Nd:YAG based PAT system. As can be seen from (2.1), on increasing the number of steps, the scan time should increase by a factor of number of steps multiplied by (ω/α) , i.e., velocity divided by acceleration. For our experiments, the acceleration was kept much larger than velocity, making the ratio of velocity and acceleration almost negligible as compared to the rest of the equation. However, this factor becomes prominent when the total scan time is less, like in PLD based PAT system, wherein the scan time increases by $\sim 60\%$ when the number of steps is increased. It should also be noted that the experimental scan time was more than the practical scan time for all the experiments. This was due to vibrations of the motor after moving each step. Motion of motor was optimized such that after each step it had to wait for a few milliseconds before starting signal collection.

It can therefore be concluded that in terms of scan time and laser exposure, continuous scans have an advantage over stop-and-go scans. This advantage becomes more prominent while using high repetition rate lasers like LED/PLD. In case of low repetition rate laser (like Nd:YAG) the improvement of scan time is apparent when less number of A-lines is collected.

2.4 Conclusion

Here, we have compared the two scanning methods that are being used for PAT/-PACT imaging systems. We have provided experimental data of phantoms as well as *in vivo* rat brain images for the comparison. The results indicate that various factors should be considered while choosing the type of scan. For smaller samples (in the range of 10–30 mm), it is better to use continuous scans, as for these samples the image quality remains quite similar for both the types of scans, and the data acquisition time is much less for continuous scans. The results also indicate that while collecting same number of A-lines for stop-and-go scans, it was better to complete the rotation in more number of steps. Increasing the number of steps improved the image quality, with only a slight increase in data acquisition time, specially for lasers with low PRR. Another factor influencing the choice of scanning method should be the PRR of the laser. Performing stop-and-go scans for lasers with high PRR exposes the samples to more number of laser pulses, which may be a safety concern. Hence, we conclude that continuous scans should be preferred over stop-and-go scans, especially during biomedical imaging of small objects using lasers with high PRR.

Chapter 3

Comparing photoacoustic imaging depth for different irradiating wavelengths²

3.1 Introduction

In photoacoustic imaging, imaging depth is scalable and is dependant on the system configuration. Unlike pure optical imaging modalities such as optical coherence tomography, two-photon microscopy, confocal microscopy, or Raman imaging which have a maximum imaging depth of a few millimeters, photoacoustic imaging has an imaging depth of few centimeters. This is because PAI is based on acquisition of US signals generated by the absorbers inside the sample and in biological tissues ultrasound scattering is approximately two orders of magnitude less than optical scattering. The generated PA signal depends on the illumination light wavelength, the optical properties of the target sample, and propagating background medium.

²Part of the work presented in this chapter has been published in *Journal of Biomedical Optics*. **A. Sharma**, Srishti, V. Periyasamy, and M. Pramanik, “Photoacoustic imaging depth comparison at 532-, 800-, and 1064-nm wavelengths: Monte Carlo simulation and experimental validation,” *Journal of Biomedical Optics* 24(12), 121904 (2019).

Light within the visible window (400 to 600 nm) is generally used to image vasculature, as blood is a strong optical absorber in the visible window [144]. However, surrounding tissues also absorb visible light, leading to poor imaging depth. The imaging depth can be increased by selecting suitable wavelengths for illumination, wherein scattering and absorption in background tissue are minimal. Therefore, first near-infrared window (NIR-I) (700 to 900 nm) is widely used for sample illumination in PAI rather than visible (400 to 600 nm) region for deep tissue imaging [38, 79, 145]. Although imaging depth is increased in NIR-I, it comes with a cost of a bulky, inefficient, expensive two-stage [Ti:sapphire, dye, or optical parametric oscillator (OPO)] laser needed to generate the light in this window. Recently, a second window of near-infrared region (NIR-II, 900 to 1200 nm) has been used for PAI [56, 129, 130, 146–149]. This increased interest in NIR-II imaging has been attributed to further decrease in scattering of light by tissue in this region, and to the increased maximum permissible exposure (MPE) of light illumination in accordance with the American National Standards Institute (ANSI) safety limit [140]. However, as compared to the NIR-I window, absorption of light by surrounding tissues is higher in NIR-II. The combined effect of decreased scattering, increased absorption, and increased MPE on depth of imaging in biological tissues has not been compared systematically.

Here, we have done a comparative analysis of PAI depth in breast tissue in various optical wavelength ranges. For the first time, we have systematically compared and analyzed the penetration depth in tissue using Monte Carlo (MC) simulations and have further validated them experimentally. We compared different scenarios to account for change in imaging depth due to change in target sample, MPE at different wavelengths, and imaging modality. Since vasculature imaging and SLN imaging using external contrast agent [150, 151] are two common applications of PAI, environments mimicking these scenarios were considered. Different chromophores present in the two environments helped evaluate the effects of absorption coefficient (μ_a) of target sample on imaging depth. Further, by analyzing the results at both constant energy and MPE, relative effect on imaging depth due

to change in MPE and change in tissue optical properties at different wavelengths has been examined.

MC simulations were first performed to simulate light propagation in human breast tissue with embedded targets (mimicking blood vessel or SLN) at wavelengths 532, 800, and 1064 nm. Owing to ease of availability, 532 and 1064 nm are extensively used for PAI. No considerable change in optical properties is present within NIR-I; therefore, the penetration depth remains similar in the entire window [152, 153]. Since 800 nm has been used for breast vasculature imaging, [154] it was considered an ideal choice for performing simulations. Another consideration for selecting the wavelengths was availability of tissue optical properties in a single publication in all the three optical windows. Therefore, for comparing penetration depths in visible, NIR-I, and NIR-II regions, wavelengths of 532, 800, and 1064 nm were selected, respectively.

The simulation results were then validated by performing experiments on both PAT and acoustic resolution photoacoustic microscopy (AR-PAM) systems using chicken breast tissue sample and blood/ink targets. Wavelengths of 532, 740, and 1064 nm were employed for sample illumination in PAT. Wavelengths used for experiments were slightly different from those used for simulations, due to system restrictions. However, since changes in optical properties [i.e., μ_a , scattering coefficient (μ_s), scattering anisotropy (g)] of blood and tissue between 740 and 800 nm are minimal, simulation and experimental results were expected to align. For AR-PAM, samples were illuminated with 570- and 1064-nm wavelengths. The depth of imaging and the signal-to-noise ratio (SNR) were studied for all the experiments.

FIGURE 3.1: (a)–(c) Cross-sectional view of simulation geometry at $y=0$ during MC simulations for (a) uniform tissue medium, (b) tissue with cylinder mimicking blood properties placed with center at depth d from surface, and (c) tissue with sphere mimicking constant absorber placed with center at depth d from surface. (d)–(f) Results of MC simulation corresponding to geometries (a)–(c) showing (d) fluences across depth for different wavelengths, (e) absorbance in cylinder, and (f) absorbance in sphere. (g)–(i) Results obtained for (d)–(f) after multiplication with factors proportional to MPE. Symbols for (d)–(i) are same as shown in (d).

3.2 Materials and Methods

3.2.1 Monte Carlo Simulations

MC simulation for light propagation in multilayered tissue (MCML) with embedded objects (MCEO) was modified to compute the fluence in tissue across depth and the absorbance in objects embedded in tissue at different depths [155]. The light propagation in the simulation geometry is determined by the optical properties of the medium such as μ_a , μ_s , g , and refractive index (η) [156]. Three different simulation geometries were used to compare the propagation of photons at different wavelengths. Figure 3.1(a)–(c) illustrate the cross-sectional view of simulation geometries at $y = 0$ plane, across xz axis. The first simulation geometry [Figure 3.1(a)] mimics human breast tissue of thickness 30 cm. The fluence across the depth of the medium was recorded for a pencil (an infinitely narrow collimated) beam. Simulations were executed for wavelengths 532, 800, and 1064 nm.

Figure 3.1(b) demonstrates the second geometry mimicking absorption of light in a blood vessel embedded in human breast tissue. A cylinder of 1 mm diameter and infinite length was placed at different depths in the tissue medium, which was subjected to pencil beam illumination. Absorbance in cylinder was computed at different wavelengths by varying optical properties of the medium and the cylinder to match the properties of human breast tissue and blood, respectively [157, 158]. Distance between the tissue surface and the cylinder was increased to compute the absorption of light by the blood vessel at different depths.

MCEO was further performed on the geometry as illustrated in Figure 3.1(c). Here, absorbance was calculated in a sphere of diameter 1 cm placed at different depths in tissue. The μ_a of the sphere was constant ($= 1 \text{ cm}^{-1}$) across all wavelengths, while its μ_s was equal to the μ_s of surrounding tissue. Such geometry mimics imaging of organs such as urinary bladder or SLN, which generally require injecting contrast agent such as methylene blue, indocyanine green, gold nanoparticles, and black ink, during imaging [159]. The sphere, therefore, mimics the organ filled with contrast

Layer	Medium (Human breast)			Cylinder (Blood vessel)			Sphere (Absorber)		
Wavelength (nm)	532	800	1064	532	800	1064	532	800	1064
$\mu_a(\text{cm}^{-1})$	0.45	0.011	0.033	224.8	3.8	4.5	1	1	1
$\mu_s(\text{cm}^{-1})$	120	116	77	727.8	767.3	592.6	120	116	77
g	0.9	0.9	0.9	0.9687	0.9833	0.9799	0.9	0.9	0.9

TABLE 3.1: Optical properties of surrounding medium, cylinder, and sphere, used for MC simulations. Refractive index (n) was 1.33 for all the mediums.

agent. Similar to the previous case, the position of the sphere and the properties of the sphere and the tissue were modified to get absorption at different depths and at different wavelengths, respectively. Table 3.1 gives the optical properties of medium, cylinder, and sphere, used for simulations.

10^8 photons were launched for each simulation to ensure high computational accuracy. The cylinder and sphere were embedded at depths 1 to 6 cm from the tissue surface in steps of 1 cm. All simulations were performed on a desktop computer with an Intel Xeon 3.7 GHz, 64-bit processor, and 16-GB RAM running windows 10 operating system.

3.2.2 Acoustic Resolution Photoacoustic Microscopy Imaging System Configuration

AR-PAM system was used for comparing the images obtained using laser illumination of 570 and 1064 nm. A nanosecond diode-pumped solid-state Nd:YAG laser (INNOSLAB, Edgewave, Wurselen, Germany) generated laser pulses of wavelengths 532 and 1064 nm at a pulse repetition rate (PRR) of 5000 Hz. A harmonic beam splitter (HBSY11) reflected a 1064-nm beam toward the 1064-nm AR-PAM system and the transmitted 532-nm beam was allowed to pass through a dye laser (Credo-DYE-N, Sirah dye laser, Spectra Physics, Santa Clara, California) toward

the 570-nm AR-PAM system. More detailed system descriptions (and performance) of the 570-nm AR-PAM system [160] and the 1064-nm AR-PAM system [56] can be found elsewhere. The PA signal generated by the sample after irradiation with the 570-nm beam was collected by a 50-MHz UST (V214-BB-RM, Olympus NDT, Waltham, Massachusetts) housed in the center of an optical condenser. Before acquisition, the PA signal was focused with an acoustic lens (LC 4573, Thorlabs) having a radius of curvature of 4.6 mm and a diameter of 6 mm, attached to the UST. For the 1064-nm AR-PAM system, a 30-MHz UST attached to a diverging lens (LC1975-C, Thorlabs) of diameter 6 mm and radius of curvature of 12.4 mm was used to acquire the generated PA signal. The UST was positioned at the center of the optical condenser to achieve confocal alignment of optical and acoustic foci.

In both the AR-PAM systems, the scanning setup was mounted on a three-axis motorized stage (PLS 85 for x and y axes, VT 80 for z axis, PI-Physik Instrumente, Karlsruhe, Germany) which was controlled by a three-axis controller (SMC corvus eco, PI miCos). The scanning head was immersed in a water tank (13×30 cm) having an imaging window with diameter of 10 cm sealed with polyethylene membrane.

3.2.3 Photoacoustic Tomography Imaging System Configuration

The system configuration (and performance) for PAT system has been described in detail in Chapter 2. A Q-switched Nd:YAG laser delivered laser pulses of wavelengths 532 and 1064 nm at a PRR of 10 Hz. These pulses were split using a harmonic beam splitter (HBSY11) before being guided toward the imaging area. For generating pulses of wavelength 740 nm, the beam from Nd:YAG laser was pumped into a tunable OPO (Continuum, Surelite OPO) laser. The desired wavelength was guided to the sample using three right-angled prisms (PS911, Thorlabs). The setup was arranged such that the sample was irradiated homogeneously by laser pulses from above. An optical diffuser was used to homogenize the beam.

The PA signal generated by the sample was collected using a 5-MHz single-element UST (V309-SU, Olympus NDT) having a 13-mm diameter active area and a 70% nominal bandwidth. UST was connected to a stepper motor (Lin Engineering, Silverpak 23C) which rotated it around continuously to collect signal from different angles. Both the sample and the UST were placed in a water tank to enable better coupling of US signal.

3.2.4 Sample Preparation

Two transparent, low-density polyethylene (LDPE) tubes having an inner diameter of 0.59 mm were filled with black ink and fresh blood, respectively. Blood was chosen owing to its vast use as an endogenous light absorber in the body. Black ink, due to its high coefficient of absorption across the visible and NIR spectrum, mimics the properties of any exogenous contrast agent used during PAI.

For AR-PAM, the tubes were placed under the imaging window of the water tank, while for PAT, they were directly placed inside the tank. Slices of different thicknesses of commercially available chicken breast tissue were stacked on the LDPE tubes to illustrate imaging at different depths. AR-PAM data were acquired by raster scanning over the tubes in an area of 8×10 mm. Maximum amplitude projection (MAP) of the acquired data was computed using MATLAB (MathWorks, Natick, Massachusetts). PAT data were acquired by rotating the UST continuously around the sample at a speed of 1.5 deg/s. The collected data were averaged into 240 A-lines to compensate for pulse-to-pulse variation of laser power. A simple delay-and-sum algorithm [161] was implemented for data reconstruction.

United States Air Force (USAF) 1951 test target (R1DS1P, Thorlabs) was further imaged to validate the depth of imaging in AR-PAM systems. The target was placed below the imaging window, and slices of chicken breast were stacked on it. The scanning head was raster scanned in an area of 20×20 mm across the test target. Transparent US gel was used as a coupling medium between the sample

Wavelength (nm)	MPE/pulse (mJ cm^{-2})*	Energy density/pulse (mJ cm^{-2}) (PAT experiments)	Multiplication factor (κ) (Simulations)
532	20	4.2	1
740	24	5.1	N/A
800	32	N/A	1.6
1064	100	21.4	5

TABLE 3.2: Per pulse fluence and multiplication factors for different wavelengths. *: maximum permissible energy for more than 10 seconds of skin exposure for 10 Hz laser.

and the imaging window. MAP of the acquired data was computed to form the final image.

3.2.5 Laser Power

Laser energy density on the surface of biological tissues is governed by ANSI safety limits [140]. The safety limit depends on incident light wavelength and time of exposure on the sample. To analyze the effect on imaging depth due to change in optical properties of tissue (which is dependent on the wavelength of illumination) and change in MPE of light independently, experiments were conducted in two ways. Initially, imaging depth was compared using constant laser fluence across all wavelengths. Later, the fluence was changed proportionally to the MPE at that wavelength.

For AR-PAM systems used here, the allowable per-pulse energy density for the 570 and 1064 nm are 1 [162] and 2.96 mJ/cm^2 [56], respectively. To analyze images at constant laser power, experiments were conducted at pulse energy of 0.30 mJ/cm^2 for both the wavelengths. Later, pulse energy of the 1064-nm system was increased to 1 mJ/cm^2 and the acquired images were compared with the images obtained previously using the 570-nm system, to compare the results when energy is proportional to MPE.

Owing to the change in exposure time, the allowable per-pulse energy density was different for PAT. In this case, initial experiments were conducted by maintaining a

constant energy density of 5.1 mJ/cm^2 across all wavelengths. Later, pulse energy of the 532- and 1064-nm pulses was altered such that it became proportional to MPE at respective wavelengths. Table 3.2 provides the MPE and the per-pulse energy densities for different wavelengths used during PAT experiments.

For MCEO results, MPE was calculated for exposure duration between 10s and $3 \times 10^4 \text{ s}$. Simulations were performed to give values of absorbance and fluence at different wavelengths and different depths. These values were multiplied with a multiplication factor (κ) proportional to MPE, to give an estimate of relative absorbance at MPE. Values of MPE and κ used during simulations are provided in Table 3.2.

3.3 Results

Figure 3.1(d)-(i) show the results of MC simulations when tissue [Figure 3.1(d) and (g)] and tissue with embedded objects [Figure 3.1(e), (f), (h), and (i)] were illuminated by a pencil beam at different wavelengths. Fluence across depth in human breast tissue for wavelengths 532, 800, and 1064 nm has been shown in Figure 3.1(d). Figure 3.1(e) and (f) show absorbance in cylinder and sphere, respectively, when they were embedded at different depths in breast tissue. Corresponding results of Figure 3.1(d), (e), and (f) at MPE are shown in Figure 3.1(g), (h), and (i), respectively. The results obtained show a drastic decrease in tissue fluence with an increase in depth for illumination with wavelength of 532 nm. The decrease in fluence resulted in a corresponding decrease in absorbance in both cylinder and sphere. This decrease can be attributed to the high μ_a and high μ_s of breast tissue in visible region compared to NIR window.

The change in absorption with depth is much lower in NIR region, as compared to visible region. It can be observed in Figure 3.1(d)-(f) that there is a higher decrease in absorption at 1064 nm illumination than at 800 nm illumination when the energy deposited for both the wavelengths is same. Although the μ_s decreases

with increase in wavelength, the μ_a in breast tissue is higher at 1064 nm than at 800 nm. This increase in μ_a results in a decrease in the amount of light reaching higher depths. Conversely, it was observed that when the tissue was subjected to exposure by all three wavelengths at MPE, fluence was highest for 1064 nm till an approximate depth of 4.5 cm, after which fluence for 1064 nm reduces compared to 800 nm. The absorbance of 1064 nm is higher than the absorbance of 800 nm, in both the cylinder and the sphere at MPE till a depth of 6 cm. Between 4.5 and 6 cm, the fluence in tissue for the 1064 nm illumination reduces with depth compared to the 800 nm, while the absorbance in the objects for 1064 nm remain higher than 800 nm. This observation can be attributed to the different properties of embedded objects at different wavelengths. Higher μ_s of embedded objects at 800 nm (compared to 1064 nm) might result in decreased absorbance of light in some parts of sphere, even though fluence in the given xy plane is larger.

It can be concluded from simulations that the fluence decreases by almost 96% per cm in visible (532 nm) region, while the average decrease in NIR-I (800 nm) is $\sim 30\%/cm$ and the average decrease in NIR-II (1064 nm) is 45% per cm. Thus, the absorbance changes by a factor of $\sim 100/cm$ in visible region and by a factor < 10 in NIR region. Further, till ~ 6 cm depth, NIR-I has higher absorbance in embedded object when laser power is same, while NIR-II has higher absorbance in embedded objects when laser power is proportional to MPE.

Experiments were conducted to validate the simulation results. Figure 3.2 shows the results obtained when LDPE tubes filled with black ink and blood were subjected to AR-PAM experiments. Figure 3.2(a)-(e) correspond to images obtained using the 570-nm AR-PAM system, while Figure 3.2(f)-(o) correspond to images obtained using the 1064-nm AR-PAM system. In the images, the tube on the left was filled with ink, while that on the right [only visible in Figure 3.2(a)-(c)] was filled with blood. Laser fluence per pulse was maintained at 0.30 mJ/cm^2 for Figure 3.2(a)-(k) and at 1 mJ/cm^2 for Figure 3.2(l)-(o).

At same fluence, the tube containing ink was visible till a depth of 4 mm in the

FIGURE 3.2: Comparison of images obtained using (a)–(e) 570-nm AR-PAM and (f)–(o) 1064-nm AR-PAM system at (a)–(k) same fluence and (a)–(e), (l)–(o) fluence proportional to MPE. Left and right tubes were filled with black ink and blood, respectively. Scale bar is same for all images as given in (a). Numbers on top of images indicate the thickness of tissue placed on the tubes. (p) The SNR for (a)–(k). Solid lines indicate ink and dashed lines indicate blood.

570-nm AR-PAM system and 5 mm in the 1064-nm AR-PAM system. Further, on increasing the surface fluence of the 1064-nm AR-PAM system such that the surface fluence for both the wavelengths were proportional to MPE at respective wavelength, the imaging depth increased to almost 10 mm. This corresponds to more than 100% increase in imaging depth between 570 and 1064 nm AR-PAM systems for black ink. However, 570 nm showed a much stronger blood absorbance as compared to 1064 nm. LDPE tube filled with blood was visible till a depth of 3 mm in the 570-nm system, while it was not at all visible in the 1064-nm system. Visibility of blood tubes in these images is poor due to a high relative contrast of

black ink. Tubes were clearly visible in Figure 3.2(a)-(c) on adjusting the color bar (images not shown). For quantification of results, SNR at different depths was computed as

$$SNR(dB) = 10 \times \log \frac{V}{N} \quad (3.1)$$

where V is the mean of PA signal (above a threshold) from an area where sample was placed and N is the standard deviation of the background noise.

Figure 3.2(p) shows SNR for ink (solid lines) and blood (dotted lines) for the 1064- (black triangles) and 570-nm (green circles and squares) systems corresponding to images in Figure 3.2(a)-(k). The average change in SNR of black ink was more (8 dB/mm) for the 570-nm system, as compared to the 1064-nm system (6 dB/mm). SNR of blood was 25-30% less than the SNR of ink for the 570 nm AR-PAM system. Owing to low SNR, blood was not visible beyond a depth of 3 mm.

FIGURE 3.3: Images of test targets acquired using (a) and (b) 570-nm AR-PAM system and (c)–(e) 1064-nm AR-PAM system when (a) and (c) no tissue; (b) and (d) 3-mm tissue; and (e) 8-mm tissue were stacked on the targets. Scale bar is same for all images as shown in (a).

Experiments were further conducted on test targets to assess the images obtained at different imaging depths. Figure 3.3(a), (b) and (c)–(e) show images obtained in

the 570- and 1064-nm systems, respectively. Chicken tissue of thicknesses 0, 3, and 8 mm were stacked on targets in Figure 3.3(a), (c); (b), (d); and (e), respectively. It was observed that when no tissue was placed on the sample, the images obtained using the 570-nm system showed better image quality, as compared to images obtained on using the 1064-nm system. This may be attributed to the different USTs used in the two imaging systems. Since the imaging depth of the 1064-nm system is higher, a transducer with lower central frequency was used for signal detection. This resulted in a decrease in system resolution [110]. It can also be observed from Figure 3.3(c)-(e) that an increase in tissue thickness results in an increase in the image artifacts. Presence of chicken tissue results in refraction of PA signal at multiple surfaces. Owing to the nonuniformity of tissue, refraction at different points across the image is different, resulting in slight changes in acoustic focus and acoustic impedance. Therefore, some parts of the image appear out of focus. With an increase in tissue thickness, slight degradation in image quality was observed. However, the structures of test targets were distinct and intact. This confirms that imaging of bigger structures such as lymph node and urinary bladder can be accurately performed at higher depths using the 1064-nm AR-PAM system; however, smaller structures with low absorbance might be difficult to discern at such depths.

Following this, experiments at wavelengths of 532, 740, and 1064 nm were conducted using PAT system. The images of LDPE tubes filled with blood and black ink are shown in Figure 3.4 and Figure 3.5. The left tube in the image was filled with black ink, while the right one was filled with blood. Figure 3.4 shows images acquired when the tubes were irradiated with constant surface fluence for all wavelengths. SNR values, computed in the same way as explained earlier, for blood (dotted lines) and black ink (solid lines) at different depths, for all three wavelengths are shown in Figure 3.4(o). The graph shows that PA signal from blood was maximum at 532 nm in the absence of chicken tissue. This is expected due to high μ_a of blood at 532 nm. An increase in depth of chicken tissue resulted in a decrease in SNR for all three wavelengths. The decrease in SNR per unit length

FIGURE 3.4: Comparison of images acquired using PAT at (a)–(d) 532-nm, (e)–(j) 740-nm, and (k)–(n) 1064-nm laser irradiation with same surface fluence. Scale bar is same for all images as shown in (a). Left and right tubes were filled with black ink and blood, respectively. Numbers on top of the images indicate the thickness of tissue placed on the tubes. (o) SNR for black ink (solid lines) and blood (dotted lines) at different depths for different wavelengths.

was similar for 532 and 1064 nm and was slightly lower for 740 nm. No particular trend in SNR variation was observed. For 532 and 1064 nm, the LDPE tubes containing black ink were discernible only till imaging depth of 15 mm, while for 740 nm, the tube was visible till 25 mm. Thus, the experimental results validated the simulation results, which suggested that at constant fluence, absorbance in constant absorber at higher depths will be maximum for NIR-I region.

Figure 3.5(a)–(l) show the images acquired for LDPE tubes when the surface fluence at different wavelengths was as given in Table 3.2. Corresponding SNR values for blood and ink at all three wavelengths are shown in Figure 3.5(m). Owing to a relatively low MPE in visible region, the SNR for blood and ink for 532 nm was

FIGURE 3.5: Comparison of images acquired using PAT at (a) and (b) 532-nm, (c)–(f) 740-nm, and (g)–(l) 1064-nm laser irradiation with surface fluence proportional to MPE. Scale bar is same for all images as shown in (a). Left and right tubes were filled with black ink and blood, respectively. (m) SNR for black ink (solid lines) and blood (dotted lines) at different depths for different wavelengths. (n) and (o) Images acquired at 1064-nm illumination when surface fluence was increased to maximum possible value. Numbers on top of the images indicate the thickness of tissue placed on the tubes.

lower than that observed in the previous case. Black ink was still visible to a depth of 15 mm, but image quality degraded due to reduced SNR. However, due to much higher MPE of NIR-II, the SNR values at 1064 nm were at least 30% higher than the SNR values at 740 nm for imaging depths between 10 and 25 mm. The imaging depth for 1064 nm increased to 35 mm at this surface fluence. For depths beyond 25 mm, the LDPE tubes were not discernible with 740 nm. Imaging of tissue <10 mm was not performed with 1064 nm in this case, as such high power could have resulted in boiling of black ink, thus damaging the tube.

As can be seen in Table 3.2, MPE for all wavelengths was higher than the surface fluence used for the experiments. The laser power was limited due to the use of OPO, which was used for generating 740-nm pulses. Energy of Nd:YAG laser could further be increased to achieve an imaging depth of 45 mm at 1064-nm wavelength, as shown in Figure 3.5(n) and (o). For all the images obtained using PAT, other image characteristics, such as resolution and spatial accuracy, remained same irrespective of the illumination wavelength used.

3.4 Discussion and Conclusion

Here, imaging depths in breast tissue for different wavelengths have been compared using both MC simulations and experiments. Two environments, one mimicking a blood vessel inside human breast tissue and another mimicking imaging of SLN following injection of a contrast agent, were considered for simulations. Tissue properties corresponding to three wavelengths, one each in visible, NIR-I, and NIR-II regions were used. Simulation results showed a severe decrease in laser fluence for visible region (532 nm) with an increase in depth of breast tissue. It was also observed that the decrease in fluence with depth was more for NIR-II (1064 nm) as compared to NIR-I (800 nm), when both were irradiated with same number of photons. At MPE, the fluence for 1064 nm was higher till an approximate depth of 4.5 cm, following which fluence of 800 and 1064 nm became equal and then fluence at 800 nm became larger. MCML simulations may further be theoretically convolved to compare penetration depth for different beam profiles; however, the trend in imaging depth for different wavelengths is expected to remain similar for all profiles.

Following simulations, experiments were conducted on both AR-PAM and PAT systems. AR-PAM experiments showed an increase in imaging depth for 1064 nm, as compared to 570 nm. The increment in depth of chicken breast was from 4 to 5 mm, when both the systems had same fluence, and from 4 to 10 mm, when fluence was proportional to MPE. To validate that the imaging depth of the 570-nm

system was low due to the wavelength and not due to the UST used, experiments were conducted by irradiating the sample at 570 nm but by using a 30-MHz UST instead of a 50-MHz UST (results not shown). The imaging depth using this setup was similar to what was observed in a conventional 570-nm AR-PAM system, as described above. Thus, confirming that the comparatively lower imaging depth was due to high μ_a and μ_s in tissue at 570 nm.

PAT experimental results validated the simulation results that the decrease in SNR with imaging depth was more for NIR-II (1064 nm) as compared to NIR-I (740 nm) when samples were irradiated with constant surface fluence. This resulted in higher achievable imaging depth in chicken breast tissue for 740 nm. Nevertheless, analogous to simulation results, the SNR for NIR-II was higher than the SNR for NIR-I, when pulse energy in both cases was proportional to MPE at that wavelength. Therefore, in this case, the imaging depth was higher for NIR-II, as compared to NIR-I. For our experiments, imaging depths in chromophores embedded in chicken breast were 15, 25, and 15 mm, respectively, for wavelengths 532, 740, and 1064 nm when irradiated by same surface fluence. The imaging depths changed to 15, 25, and 35 mm, respectively, for 532-, 740-, and 1064-nm wavelengths, when surface fluence was proportional to MPE. Although the simulation and experimental results follow similar trend, slight differences could still be observed. This is due to the differences in optical properties of human breast tissues used in simulations versus the chicken breast tissues used in the experiments. Further variation in penetration depth for all wavelengths may be observed on changing the type of tissue (e.g., muscles and liver). In addition, variation can be observed on using excitation wavelengths different from the ones that have been used in this study, even though they might be within the same optical window. One needs to use appropriate simulation or experiments to find out the imaging depth for different combination of tissue types or wavelengths.

Our results show that the imaging depth in breast tissue for 1064 nm was higher than 740 nm due to its higher MPE. Compared to visible region, NIR regions have higher imaging depths, owing to the decreased scattering. Penetration depth of

visible region may further decrease during *in-vivo* imaging due to presence of skin layer; however, this decrease is nominal for NIR regions and therefore skin layer has not been considered. Nevertheless, between NIR-I (740 nm) and NIR-II (1064 nm), the decrease in scattering is accompanied with an increase in absorption of surrounding tissues, which results in an overall decrease in fluence at higher depths. However, since the maximum skin exposure allowed for NIR-II is higher than NIR-I, the tissue can be irradiated with higher laser energy. Therefore, deep tissue imaging possible at NIR-II can only be attributed to higher illumination energy. Simulation results also showed that absorbance in sphere becomes similar for 800 and 1064 nm at an approximate depth of 6 cm. Further, absorbance in cylinder and sphere was higher in the NIR-I region than in the NIR-II region, even when illumination was proportional to MPE. Owing to the system restrictions, experimental validation of this trend was not possible as it was difficult to get enough energy density to achieve such imaging depth experimentally.

Another reason of growing popularity of NIR-II window, specifically 1064 nm, is the availability of high laser power at this wavelength. Since traditional Nd:YAG laser can directly deliver pulses of wavelength 1064 nm, there is no need of a dye laser or OPO, which is generally needed for generating wavelengths in NIR-I region. Owing to low efficiency (15% to 25% efficiency) of the second-stage laser, their presence not only increases the cost of the system but also results in a decrease in available power. For the PAT experiments illustrated above, maximum laser power of OPO could provide an imaging depth of 25 mm at 740 nm illumination, whereas maximum laser power of Nd:YAG could provide an imaging depth of 45 mm at 1064 nm illumination.

In summary, we have compared the PAI depth in breast tissue for illumination of target samples at different wavelengths. Wavelengths in visible, NIR-I, and NIR-II regions of optical spectrum were compared via MC simulations and via different experiments. We have shown that, for deep tissue imaging, a wavelength of 1064 nm can be a good option, owing to its high MPE and easy availability of high-power lasers with compact size and cheaper cost. However, as compared to wavelengths

in visible region, μ_a of blood is lower at 1064 nm. For this reason, the visible region should be preferred for vasculature imaging at lower depths. Contrast agents are generally needed in NIR region due to low μ_a of blood at those wavelengths. Both blood and ink showed higher absorbance at deeper depths in breast tissue by 1064-nm (NIR-II) illumination compared to 740-nm (NIR-I) illumination at MPE. Therefore, we conclude that for imaging depths <6 cm in breast tissue, 1064 nm (NIR-II) seems more promising than 740/800 nm (NIR-I) for PAI.

Chapter 4

Convolutional neural network for resolution enhancement in acoustic resolution photoacoustic microscopy³

4.1 Introduction

Imaging resolution is an important characteristic while defining any biomedical imaging system. Among various modalities of PAI, photoacoustic microscopy is commonly employed to get high resolution images. In photoacoustic microscopy (PAM) focused optical illumination and/or focused ultrasound detection is used to obtain high resolution images at depth beyond optical mean free path [35]. For pre-clinical applications requiring high resolution images, AR-PAM is a common choice as it provides images beyond the optical diffusion limit [61]. AR-PAM employs a weakly focused (or unfocused) light illuminating the sample, and a tightly focused

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ultrasound transducer (UST) acquiring the generated PA signals [108]. The lateral resolution of AR-PAM system is determined by the properties of UST and is given by

$$LR_{AR-PAM} = \frac{0.71\lambda_a}{NA_a} = \frac{0.71\nu}{NA_a \times f_c} \quad (4.1)$$

where λ_a is the center wavelength of the detector, NA_a is the acoustic numerical aperture ν is the speed of sound, and f_c is the central frequency of the UST [29, 163]. Lateral resolution can be improved by increasing the central frequency and the NA of the UST. However, high frequency ultrasound waves attenuate more in biological tissue and therefore, decrease the imaging depth [111, 164]. Increasing the NA of the UST results in improved lateral resolution, but it reduces the focal zone [92]. This leads to degradation of lateral resolution outside the focal plane.

Synthetic aperture focusing technique (SAFT) using the concept of virtual detector was first introduced to improve the out-of-focus resolution in AR-PAM [165]. This method generates new time-resolved PA signals (A-lines) by adding delayed versions of original A-lines, which originated from the same source. However, a discrepancy between actual PA wavefronts and estimated PA signal has limited this enhancement of image resolution. Further modifications of SAFT like 2D-SAFT [135], adaptive SAFT [166], weighted SAFT [136], and SAFT with deconvolution [167] have been proposed. In addition, different types of beamformers are also used in AR-PAM. Delay-and-sum (DAS) is a common beamformer used due to its ease of implementation. Improvement in images has been shown by using delay-multiply-and-sum (DMAS) algorithm [168].

In our previous work [169], we showed that double-stage delay-multiply-and-sum (DS_DMAS) can outperform DAS and DMAS in enhancing AR-PAM images. DS_DMAS was used as a beamformer and implemented along with SAFT algorithm. Use of DS_DMAS improves the contrast of the resultant images by decreasing the noise level. We showed that DS_DMAS + SAFT can reduce the noise level

for all depths by about 134%, 57% and 23%, compared to the original low resolution, SAFT+DAS and SAFT+DMAS methods, respectively. Although these algorithms improve the out-of-focus resolution, they generally suffer from limitations like lower signal to noise ratio (SNR), high post-processing time, or dependence of improvement on orientation of the sample.

Here we propose a deep learning (DL) based approach to improve primarily the lateral resolution of out-of-focus AR-PAM images, with a corresponding increase in PA signal strength and decrease in background noise. Advancement in computing techniques has led to rapid growth of artificial intelligence (AI). Among different AI techniques, DL has particularly become popular due to its ability to get trained and learn from pre-defined dataset [170–172]. DL uses multilayered artificial neural networks to enable supervised learning. Computer vision, the branch of DL associated with image analysis and classification, commonly employs convolutional neural network (CNN) for this purpose. CNNs use a convolution layer with a non-linear operator to extract essential features of an image by learning weights and filters [173, 174]. Last few years saw a significant growth in application of DL for image classification and processing. DL has been used for image enhancement of ultrasound images [175], MRI [176], PET [177], CT [178] etc. Deep learning methods have also been used for reconstruction and enhancement in photoacoustic imaging [179–184].

A simple deep neural network was trained to increase the bandwidth of detected PA signal and improve the quantitative accuracy of acquired PA images [185]. Following this, CNNs were used for reconstructing PA images from pre-beamformed data [186] and from under-sampled data [182, 187, 188]. Moreover, correction of motion artefacts [189], removal of reflection artefacts [190], and deblurring [191] of PA images has also been done using CNNs. Among different architectures, there has been an increased popularity of U-Net based-architectures due to their advantages of being fast and providing promising results on segmentation and reconstruction of medical images even when trained with limited dataset [192, 193]. Reconstruction of PA images from sparse data has been demonstrated by following

filtered backpropagation algorithm with a trained U-Net architecture [194]. This network was further expanded by including a dynamic aperture length correction algorithm to decrease under-sampling artifacts and blurring of PA images [195]. Following this, fully dense U-Net (FD-U-Net) was developed and demonstrated to be superior in performance for removing artifacts and denoising PA images [196]. Modified FD-U-Net has also been shown to be computationally efficient for reconstructing and removing artifacts of PA images from sparse and undersampled data in various types of PA systems [182, 187, 197]. Recently, application of U-Net combined with a fully connected network for vascular segmentation of PA images was also shown [198].

In this work, we propose a U-Net based CNN architecture to improve out-of-focus lateral resolution of AR-PAM images. So far in the literature no one has applied a U-Net based architecture for improving resolution of AR-PAM images. A fully dense U-Net (FD-U-Net) [187, 196] was trained with simulated (using k-wave toolbox of MATLAB [199]) AR-PAM images. The performance of the network was tested on phantom as well as *in vivo* rat vasculature imaging. The CNN-based images showed negligible change in resolution across the entire imaging depth. Moreover, compared to original PA images, up to 250% increase in signal strength and 100% decrease in background noise in out-of-focus regions was observed in CNN-based images.

4.2 Materials and Methods

4.2.1 Network architecture

U-Nets generally consist of a contracting-, a bridge-, and an expanding- path and produce an image the same size as the input [192]. For each layer in the contracting path, the image size is decreased by a factor of 2, while the number of channels is doubled. Reverse is done in the expanding path to symmetrically modify the spatial dimensions of the output. Loss of important features during contracting is

FIGURE 4.1: Schematic of fully dense (FD)-U-Net architecture employed for training. Numbers in left column indicate size of image in that row. Numbers of top-left corner of a block indicate number of channels in that block. Subset of the image shown in the yellow dashed box shows the architecture of FD-Block* used in each layer. BN: Batch normalization; ReLU: Rectified Linear Unit.

prevented by adding skip connections between contracting and expanding paths. The network therefore learns relevant features at various spatial scales, and simultaneously reduces the artefacts. Although a basic U-Net model can effectively perform medical image segmentation, a denser network is preferred for higher level image processing. Addition of a fully dense (FD) block in each layer makes the network dense thus allowing it to learn additional feature maps. By increasing the number of trainable parameters from ~ 8 to ~ 18 million, this FD-U-Net permits the network to learn more information. Dense connectivity also alleviates learning of redundant features and improves the flow of information. Therefore, a fully dense U-Net (FD-U-Net) similar to the one proposed in [187, 196] was trained for this work.

Figure 4.1 shows the network architecture used in our model. Briefly, the proposed CNN architecture consisted of 5 layers each in contracting and expanding paths and 1 bridge block. 1×1 convolution was present in each layer to learn the features.

Downsampling and upsampling were attained via 3×3 convolution and deconvolution, respectively, with a stride of 2. Each layer had an FD block designed to double the number of channels by first learning f/k feature maps (for f initial channels) via convolution, and then concatenating them with the input. Convolution of the concatenated output again produced an output with f/k channels. Repeating the process k times yielded $2f$ channels. For this network, k was equal to 4. Subset (yellow dashed box) in Figure 4.1 shows different layers of FD block. Convolutions and deconvolutions were followed by batch normalization [200] and rectified linear unit (ReLU) activation [201]. 70% dropout was added in the FD layer for regularization of the network [202]. Contracting and expanding layers were joined using skip connections which also enhanced network training by decreasing the probability of vanishing gradient. Following the expanding path, a 1×1 convolution yielded an output of the same size as the initial image. Adding this output to the initial input image and performing ReLU activation resulted in the final image. This was done to facilitate residual learning i.e., the network learned R where $ReLU(R + X) = Y$. It was easier for the network to learn R rather than Y , hence enabling faster training of the network [196]. Compared to the previously developed FD-U-Net, 3 modifications were made in this network. (1) Addition of layers at the beginning and end of the network allowed the number of pixels to be 256×256 , which thereby helped in maintaining the pixel size to be smaller than system resolution; (2) presence of dropout layer aided in regularization of network, thus preventing overfitting; (3) ReLU activation in the end enabled faster training, as the ground truth images (Y) contained non-negative values only.

During training, 10% of the images were randomly selected as test dataset. The remaining images were split in a ratio of 8:2 for training and validation, respectively. The model was trained keeping a batch size of 8, Adam optimizer with its default values (learning rate = 0.001, beta_1 = 0.9, beta_2 = 0.999, epsilon = 10^{-07}) as optimizer, Glorat normal (Xavier normal) weight initializer, and mean squared loss as the loss function. Over-fitting of data was prevented by applying early stopping callback. Loss on validation set was monitored and the training

was stopped once the loss stopped improving. The training was implemented on Python (version 3.7.6) using Tensorflow (2.3.0) framework [203] and Keras [204] library running on GPU (Nvidia V100-PCIE-16GB) using the nodes of Gekko cluster, high performance computing center (HPC), Nanyang Technological University, Singapore.

4.2.2 Image acquisition in AR-PAM

In a typical AR-PAM system, a tightly focused UST detects the PA signal generated due to irradiation of sample by a loosely focused optical beam (dark field illumination). Irradiation of the sample by a single pulse results in generation of an A-line (one-dimensional PA signal along the depth direction, say along z-axis). Multiple A-lines were acquired by sweeping the confocal alignment of the optical beam and UST along one axis parallel to the object (say y-axis). 2-dimensional absorption map (z-by-y axis), known as a B-scan, was formed by placing the A-lines next to each other. Multiple B-scans were collected by raster scanning method, (i.e., moving the UST in steps along the x-axis) to form a 3-dimensional PA image. After acquisition of A-lines, 3D volume rendering or 2D projection images [i.e., maximum amplitude projection (MAP)] were used to visualize the PA images.

4.2.3 Curation of synthetic dataset

For supervised learning, a large dataset (acquired images and their ground truth) is required for training. Here, synthetic dataset was generated by simulating B-scans using k-wave toolbox in MATLAB [199]. Figure 4.2 shows the geometry used for simulations. A 16×16 mm grid with a pixel size of $6 \mu\text{m}$ was used. An arc-shaped 6 mm sensor having a radius of curvature of 4.6 mm and a focal length of 10 mm, moved parallel to the y-axis to collect A-lines. The sensor had a 50 MHz center frequency and 70% bandwidth and was designed to mimic the UST of the AR-PAM system used for experimental validation [162]. The UST traversed 10 mm with a

FIGURE 4.2: Schematic of grid used during simulations showing area of sources (red shaded region) and different positions of the ultrasound transducer (UST).

step size of $36\ \mu\text{m}$ collecting one A-line (time step = 8 ns) at each position. B-scan was formed by stacking these A-lines next to each other and taking the absolute values of their Hilbert transformation. Sources were formed by randomly placing multiple objects at a distance of 5 mm above and below the focal plane (shaded region in Figure 4.2) of the UST. Four different geometries (points, circles, discs, and lines) of sources of sizes between 6 and $180\ \mu\text{m}$ were used to mimic cross-section of different biological samples. Initial pressure rise of the sources was varied, and noise was added in some images such that the SNR was 30, 20, or 10 dB. For all the simulations, the medium was assumed to be homogeneous with 1500 m/s sound speed. All simulations were performed on GPU (Nvidia V100-PCIE-16GB) using nodes of Gekko cluster, HPC, Nanyang Technological University, Singapore.

The ground truth images of source position and their corresponding B-scans were both downsampled such that the final pixel size in both directions was $36\ \mu\text{m}$. Following this, the source image was normalized, and the generated B-scans were scaled accordingly. Some source images along with their generated B-scans were

FIGURE 4.3: Examples of training data containing sources in shape of (a, b) discs, (c, d) points, (e, f) circles, and (g, h) lines. During training (a, c, e, g) simulated B-scans were given as network input, and (b, d, f, h) their ground truth images as desired (true) output. Scale bar is shown in (a).

further decreased in intensity by randomly multiplying with a factor ξ ($0.4 < \xi < 1$). Range of ξ was chosen to include weaker PA vasculature signal while removing background noise. This was done to bridge the gap in PA signal amplitude between simulated and experimental PA images. Using different combinations of type, number, position, initial pressure rise of sources, and different noise levels, 2095 B-scans were generated for training (1508), validation (377), and testing (210). These processed B-scans were considered as the input images (X) while the source images were considered the desired output or the ground truth (Y) during training of the neural network. Figure 4.3 shows examples of input B-scans and their corresponding ground truth images. Sources of different shapes [e.g., discs (Figure 4.3(b)), points (Figure 4.3(d)), circles (Figure 4.3(f)), and lines (Figure 4.3(h))], size, and initial pressure rise were randomly placed to generate B-scans [Figure 4.3(a),(c),(e),(g) respectively]. Background noise was also added in some images as shown in Figure 4.3(c), where SNR was 10 dB. The simulated B-scans were all used as test image, while the images of the source were their respective ground truth images.

4.2.4 Evaluation Parameters

During model training, its performance was monitored on simulated data before testing it on experimental PA images. Mean absolute error (MAE) and mean squared error (MSE) for both the training and the validation dataset were the regression metrics calculated during training. MAE computes the average of the absolute values of difference while MSE computes the average of the squared difference between the ground truth and the CNN-based image i.e.,

$$MSE = \frac{1}{n} \sum_{i=1}^n (Y_{true} - Y_{out})^2 \quad (4.2)$$

$$MAE = \frac{1}{n} \sum_{i=1}^n |Y_{true} - Y_{out}| \quad (4.3)$$

Where, n is the total number of images, Y_{true} is the ground truth image, and Y_{out} is the model output (CNN-based) image. A decreasing MAE ensures that the difference between the CNN-based and the ground truth is decreasing. MSE gives more weightage to larger errors, thereby decreasing the outliers in the images. By giving higher error for larger mistakes, MSE suppresses large variations between output and ground truth, and hence it was the loss function during training.

Post training, MAE, MSE, structural similarity index (SSIM), and peak signal-to-noise ratio (PSNR) between the network output and ground truth images of test dataset were calculated. SSIM and PSNR were calculated using default TensorFlow equations. SSIM computes structural similarity between two images based on their luminance, contrast, and structure [205]. PSNR computes the peak signal-to-noise ratio between two images such that

$$PSNR = 10 \log_{10} \frac{(L - 1)^2}{MSE} \quad (4.4)$$

where L is the number of maximum possible intensity levels in an image and MSE is the mean squared error. Higher values of SSIM and PSNR denote higher resemblance between the output and ground truth images. Multiple models containing different layers and hyperparameters were evaluated. The model which delivered best performance on these parameters on test dataset was chosen as the appropriate model and was tested on the experimental PA images.

4.2.5 Experimental AR-PAM imaging system

Performance of the trained model was analyzed on experimental AR-PAM images. The imaging system is a dark field AR-PAM system mentioned in Chapter 3. It uses a diode-pumped solid-state Nd-YAG laser (INNOSLAB, Edgewave, Wurserlen, Germany) combined with a dye laser (Credo-DYE-N, Sirah dye laser, Spectra Physics, Santa Clara, CA, USA) to generate 570 nm laser pulse at a pulse repetition rate of 5 kHz. After passing the laser beam through a variable neutral density filter (NDC-50C-4M, Thorlabs), it was focused on a multimode fiber (M29L01, Thorlabs) using an objective (M-10X, Newport, Irvine, CA, USA). The output beam from the multimode fiber was collimated (LA1951, Thorlabs), and then converted to a ring-shaped beam by passing through a conical lens (1-APX-2-B254, Altechna, Vilnius, Lithuania). A confocally aligned UST (V214-BB-RM, Olympus-NDT, Waltham, MA, USA), having a central frequency of 50 MHz and a 70% bandwidth, housed at the center of the optical condenser acquired the PA signal. An acoustic lens (LC4573, Thorlabs) with radius of curvature of 4.6 mm, 6 mm diameter, and 10 mm focal length was attached to the UST to provide an acoustic focal spot of $\sim 46 \mu\text{m}$. This scanning set-up was mounted on a 3-axis motorized stage by a 3-axis controller. A water tank placed right below the scanning head housed the phantoms used in the experiments. A 10 cm imaging window in the tank sealed with polyethylene membrane provided passage for transmission of ultrasound waves from under the tank to the UST, during *in vivo* imaging. PA signals acquired by the UST was amplified by two mini-circuit amplifiers (ZFL-500LN, Mini Circuits) having a gain of 24 dB each, before being stored by a data acquisition (DAQ) card

(M4i.4420, Spectrum, Germany) installed in a desktop. Data was acquired at a sampling rate of 250 Megasamples/second by performing two-dimensional raster scanning of imaging head using LabView as an interface. During raster scanning, step size between two A-lines was 3 μm . Following data acquisition, absolute value of Hilbert transformation of stacked A-lines was taken to generate B-scans. These were resized, cropped, and zero padded to make their dimensions same as that of training dataset. The trained CNN model was applied on the resultant B-scans to generate CNN-based PA images. Model performance was analyzed by acquiring MAP of the original and CNN-based B-scans and smoothening them by applying maximum and Gaussian filters using SciPy (Python).

4.2.6 Phantom preparation

To study the effects of training model on samples present at various distances from the focal plane, phantom experiments were conducted. Seven horse hairs (diameter $\sim 80 \mu\text{m}$) were placed horizontally in the water tank at different depths for imaging. Another phantom was prepared by embedding six such horse hairs in agar phantom. The phantoms were kept in the water tank directly below the scanning head. B-scan image containing all the hairs was obtained by moving the translation stage horizontally, perpendicular to the direction of the hair samples. Multiple B-scans were collected by raster scanning of the stage to obtain 3-dimensional and MAP PA images.

4.2.7 *In vivo* rat vasculature PA imaging

All the animal experiments were conducted in accordance to the guidelines and regulations approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University, Singapore (Animal Protocol Number ARF-SBS/NIEA0331). A 75 g adult female Sprague Dawley rat was anaesthetized by intraperitoneal injection of 0.15 mL cocktail containing ketamine and xylazine of

dosages 85 and 15 mg/kg, respectively and maintained in anaesthesia by providing vaporized isoflurane (1.2 L/min oxygen and 0.75% isoflurane, Medical Plus Pte Ltd, Singapore) via a custom-made nose cone. A pulse oximeter clipped to its forearm measured its vitals. Vasculature of the region surrounding the sentinel lymph node was imaged. Prior to imaging, a commercially available hair removal cream was applied to deplete the hair from the imaging region. The rat was placed on its side directly below the polyethylene membrane of the water tank. Transparent ultrasound gel between the imaging window and the rat prevented any air gaps. To evaluate network performance for both, two sets of PA images of the same area were collected. Initially, the rat was placed such that most blood vessels were in the focal plane. Following image acquisition, distance between the UST and the rat was increased, thereby moving the blood vessels outside the focal plane of the UST. An overdose of pentobarbital was injected in the rat to euthanize it after image acquisition.

4.3 Results and discussion

4.3.1 Performance of the network on simulated AR-PAM images

The performance of the trained FD-U-Net model was evaluated on test data set to confirm that the model had been trained. Figure 4.4 shows (a) a simulated B-scan, (b) the ground truth, and (c) the CNN-based image when (a) was fed into the model. This simulated B-scan was similar to the B-scans used for our phantom experiments and was not a part of training dataset. The similarity between the ground truth and the CNN-based images validated that the model had been trained. CNN-based and ground truth images of the simulated data were also quantitatively analyzed to evaluate model performance. Figure 4.4(d) and (e) show the variation in MAE and MSE, respectively, for different epochs during model training. As shown, errors for both the training and the validation dataset decreased

FIGURE 4.4: Results of model training on simulated data. (a) A test input B-scan fed to the model. (b) The ground truth (source) image of (a). (c) Image of (a) after model application. (d, e) Graph depicting (d) mean absolute error (MAE) and (e) mean squared error (MSE) for training and validation set for each epoch.

and converged with an increase in number of epochs. Training of the model was stopped after saturation of MSE loss of the validation set, which happened after 147 epochs and took ~ 75 minutes for the given model.

Table 4.1 shows the evaluation parameters obtained when original and CNN-based images of the test dataset were compared with ground truth images. On the test dataset, MAE and MSE were 0.0171 and 0.7009, respectively. Such low values of MAE and MSE ascertained that model had been trained. Further assessment was done by computing SSIM and PSNR. Average SSIM between ground truth and CNN-based image of the test data was 99.88% compared to just 38.44% between the simulated B-scans and the ground truth images. A corresponding improvement in PSNR from 29.19 between the simulated and the ground truth image to 52.8 between the CNN-based and the ground truth images was also observed.

Prior to testing on FD-U-Net, the images were also trained on a simple U-Net

	MAE	MSE	PSNR	SSIM
Simulated B-scan	13.05	49.68	29.19	38.44%
FD-U-Net with max pool	0.0209	0.9135	50.62	99.84%
FD-U-Net with convolution	0.0171	0.7009	52.8	99.87%

TABLE 4.1: Values of the evaluation parameters between ground truth and simulated/CNN-based images on test dataset.

based architecture. Although this network was able to produce a binary mask determining whether signal is present or not, it did not perform well in inferring the amplitude of PA signal. In U-Net, the validation loss saturated when MSE was greater than 300. Therefore, the model was modified to include fully dense layers to enable better training. To further evaluate effects of different layers, max pooling was used for downsampling in the contracting layer, instead of convolution with stride 2. Although the resultant model exhibited training in the similar manner as was depicted by our FD-U-Net model, the final MSE, SSIM, and PSNR values on testing set were slightly worse (0.91, 99.8%, and 50.62, respectively), therefore, convolution layer with stride 2 was preferred for downsampling. Hence, multiple layers and architectures were optimized, trained, and tested on simulated data, before being examined on experimental B-scans.

4.3.2 Performance of the network on experimental AR-PAM images

Figure 4.5 shows the AR-PAM images of different horse-hair phantoms. Initially the hairs were placed directly in water such that the leftmost hair was deepest, and the rightmost hair was shallowest. The middle (4th) hair was in the focal plane. In the original MAP image [Figure 4.5(a)], both the resolution and the SNR degraded on moving away from the focal plane i.e., the fourth hair was captured very sharp while the others were comparatively blurred, and had a weaker PA signal, even though they all had similar geometries and absorbance originally. MAP

FIGURE 4.5: CNN-based AR-PAM imaging on hair phantom. (a) Original and (b) CNN-based maximum amplitude projection (MAP) images of horse hair placed in water. (c), (d) B-scans along the dotted lines in (a), (b), respectively. [e(1-3)], [f(1-3)] zoomed in images of dotted boxes in (c), (d). (g) Original and (h) CNN-based MAP images of horse hair embedded in agar phantom. Scale bar of (a-d, g,h) is shown in (c) and of (e), (f) in (e). (i) resolution, (j) signal and noise strength at various distance from focal plane for both original (squares) and CNN-based (circles) images. Fitted curves are also shown.

image obtained from CNN-based B-scans [Figure 4.5(b)] validate the improvement in resolution. B-scans along the dotted line in Figure 4.5(a), (b) are shown in Figure 4.5(c), (d), respectively. Figure 4.5(e(1-3)), (f(1-3)) show the zoomed in images of the dashed boxes in Figure 4.5(c), (d), respectively. The images showed an improvement in resolution even when the hair is at the focal plane. This may be attributed to the perfect ground truth images used during the model training. The B-scans further substantiate the degradation of resolution on moving outside the focal plane of original images, and the improvement in resolution after FD-U-Net network was used on the images. Figure 4.5(g), (h) show the original and CNN-based image of phantom prepared by embedding horsehairs in agar phantom. During this experiment, the focal plane was between the second and the third hair from the left, and the depth of hair increased and decreased on moving left and

right, respectively. Similar trend as above was observed in original and CNN-based PAM images.

Experimental results were quantified by measuring resolution and signal strength. Resolution along different depths was measured by taking the full width at half maximum of the derivative of the dashed line given in Figure 4.5(a), (b) at the edge of the hair (derivative of edge spread function gives line spread function). Figure 4.5(i), (j) show the resolution and PA signal and noise at different depths, respectively, for both original and CNN-based images. Resolution degraded by $\sim 180\%$ on moving ~ 2.2 mm (between first and fourth point) out of focus in original PA image [Figure 4.5(c)], while a variation of only $\sim 10\%$ in resolution was present in different hairs in CNN-based image [Figure 4.5(d)]. It should be noted that even though in the B-scans it appeared that the resolution decreased in the focal plane, the line spread function does not show that. This may be due to weak PA signal surrounding the target, which although appears large in the given colormap, does not impact the resolution. An improvement in axial resolution was also observed in B-scan images. However, since expected axial resolution of the system (~ 33 μm) was less than the pixel size, axial resolution was not characterized here. Figure 4.5(j) shows decrease in signal strength in both the original and the CNN-based images Figure 4.5(g), (h)) on moving out of focus, however, the decrease was just $\sim 19\%$ in CNN-based images compared to $\sim 75\%$ in original images. Up to 250% increase in signal strength (in right-most hair) was observed in CNN-based images. Additionally, a 100% decrease in background noise increased the SNR further. It is to be noted that background noise was absent (i.e., = 0) in the CNN-based images, hence the SNR could not be computed.

Comparison of original and CNN-based images showed that such model can improve out- of-focus resolution of PA images while simultaneously enhancing the signal strength and decreasing the background noise. This is better than existing VD-based algorithms where usually improvement in resolution is accompanied by signal degradation or noise addition.

FIGURE 4.6: CNN-based ARPAM imaging of *in vivo* rat vasculature showing (a, b) original MAP acquired keeping the mouse in such a way that the blood vasculature was (a) in focal plane, and (b) out-of-focal plane. (c, d) MAP obtained after passing the B-scans through the CNN. (e-g) B-scans along the dotted line shown in (a-d), respectively. Scale bar is shown in (a).

4.3.3 Performance of the network on *in vivo* vasculature images

Figure 4.6 shows original and CNN-based *in vivo* images of rat vasculature. MAP images acquired in focus and out of focus are shown in Figure 4.6(a) and (b), respectively. Similar to phantom experiments, for *in vivo* experiments also, the image became blurred when the rat was moved away from the ultrasound focal plane. Figure 4.6(c) and (d) show CNN-based images corresponding to Figure 4.6(a) and (b), respectively. The blood vessels in focus (marked by blue arrows in Figure 4.6(a), (c)) did not change prominently between the original and the CNN-based images. However, resolution of these vessels deteriorated on moving out-of-focus (blue arrows in Figure 4.6(b)). Improvement in resolution was observed on applying the

DL model on these out-of-focus images (blue arrows in Figure 4.6(d)). Moreover, the signal strength of some vessels of out-of-focus region also improved in the CNN-based images, as shown by green arrows in Figure 4.6(b), (d). Hence, applying the DL model led to a significant improvement in resolution and signal strength along with a substantial decrease in the background signal.

B-scans along the dotted lines in Figure 4.6(a)-(d) are shown in Figure 4.6(e)-(h), respectively. B-scans confirmed the decrease in background signal in CNN-based images. After applying DL model, most of the blood vessel signal (as shown by yellow arrows in the B-scans) was resolved as required. Further, some of the skin signal (shown by green dashed boxes in Figure 4.6(e), (f)) present in B-scan images also got eliminated. However, due to elimination of background signal, some of the weaker blood vessel signal also decreased, as pointed out by pink arrows in Figure 4.6(a), (c). Including the light illumination pattern during B-scan simulation may help in preventing this, as discussed later. Comparison between the original and CNN-based images validated that deep learning can enhance *in vivo* AR-PAM images by improving the resolution outside the focal plane and decreasing the background noise. Furthermore, weak skin signal can be added as an image source during B-scan generation to accurately train the model to differentiate between unwanted skin signal and important vascular signal. Including more shapes and types of objects in the training data may further make the model more versatile and improve its performance.

Testing the model on experimental images proved the network's ability to efficiently enhance the resolution, improve the signal strength, and decrease the background noise of out-of-focus AR-PAM images. These results demonstrate that DL is a promising tool for post-processing AR-PAM images. However, one major limitation of employing DL is in obtaining dataset for training. By simulating AR-PAM images, we waived the need of ground truth of experimental data, (which are difficult to obtain, especially for *in vivo* experiments). However, simulating the AR-PAM images was time consuming and required computational resources. This

limits the way step size or pixel size were chosen. Generation of one simulated B-scan in k-wave took ~ 2 hours using GPU of the HPC. Decreasing step size would have resulted in a corresponding increase in simulation time. Here, same pixel size was maintained via downsampling which led to degradation of lateral resolution from $\sim 50 \mu\text{m}$ to $\sim 80 \mu\text{m}$ in focal plane. Decrease in pixel size may help in precise characterization of both axial and lateral resolution. This can be attained by decreasing the step size of UST during simulations and increasing the input and output image size during training (i.e., making input/output image size 512×512 instead of 256×256). Moreover, increasing the number of training dataset may have improved the performance further, however, that would have led to further increase in simulation time. Additionally, all the simulations were done in 2D in this work. Doing a 3D simulation for the training may improve the efficacy of these types of algorithm even further, but with a heavy computational overhead. In the CNN-based MAP images, some places had a discontinuity in vasculature structure which was not present in the original MAP images. Such artifacts may get resolved by training the network on volumetric images containing both sources and UST in 3D. It may also be noted that although simulation and training was done only on B-scans, an improvement in resolution was observed even in perpendicular direction. This may have been due to removal of weak PA signal which improves the image resolution. Nevertheless, further optimization, simulation, and training, specially using 3D models needs to be done to homogeneously improve resolution in all directions.

Another limitation of this work was the absence of light illumination pattern during simulations. Although application of DL improved the signal strength in out-of-focus regions, there is still room for further enhancement. In AR-PAM, decrease in PA signal in moving away from focus is due to the combined effect of decrease in ultrasound detection sensitivity and optical fluence decrease. In this deep learning model, only the ultrasound signal part was accounted for, hence a decrease in signal strength on moving outside the focal plane was expected due to the light fluence variation at different depth in the medium. To compensate for the fluence

variation, it is necessary to incorporate light illumination patterns during the simulation. Furthermore, a decrease in signal strength was also observed for some weak but focused vasculature signal. Further optimization of range of values of initial pressure rise during simulation needs to be done so that the network can be precisely trained to differentiate between weak vasculature signal and noise. Performing simulations including light pattern (dark field illumination) and optimizing the range of initial pressure rises will be a part of our future work. Hence, obtaining simulated B-scans containing illumination pattern, sources of various shapes and sizes, and larger number of A-lines can help in further image refinement. We believe that by modifying the simulated images and algorithms, DL has the potential to simultaneously enhance the images further by increasing the contrast to noise ratio, decreasing reflection artefacts, and removing skin signal.

4.4 Conclusion

Here, a fully dense U-Net model was trained on simulated AR-PAM images to improve the image resolution outside the focal plane. The trained model was tested on experimental phantom images as well as *in vivo* rat vasculature PA images. The original phantom images showed $\sim 180\%$ degradation in resolution on moving outside the focal plane. Application of DL nullified the degradation and provided almost constant resolution across the entire depth of imaging (~ 4 mm). The *in vivo* experiments further validated the improvement confirming that the model can perform well on vasculature imaging. Moreover, 100% decrease in background noise and up to 250% increase in PA signal were observed in experimental images after application of DL model.

Chapter 5

Application of PAT for imaging chicken embryo in bioengineered eggshell⁴

5.1 Introduction

Chicken embryo has been a model organism for developmental biology for a very long time [206–208]. It is expansively studied in fields of developmental biology [209], cardiovascular studies [210], and angiogenesis [211, 212]. Research in chicken embryos has led to many fundamental breakthroughs in developmental and cancer biology including knowledge of formation and septation of heart, discovery of viral oncogenes and identification of transcription factors [210, 213–215]. Experimental techniques for studying chicken embryo are well established and various methods have been reported for visualizing the embryo. Imaging the embryo through a window in the eggshell is a common method [216–218], but the small window size limits the view. This is overcome by transferring the embryo into a petri

⁴Part of the work presented in this chapter has been published in *Journal of Biophotonics*. **A. Sharma**, N. Ishak, T. Swee-Hin, and M. Pramanik, “High resolution, label-free photoacoustic imaging of live chicken embryo developing in bioengineered eggshell,” *Journal of Biophotonics* 13(4), e201960108 (2020).

dish [219] or explanting [220] the embryo before imaging, which implies that the embryo needs to be sacrificed post-imaging. *Ex ovo* cultures using weigh boats [221], polyurethane membrane hammocks [222], or surrogate shells [223] have been efficiently used for studying growth of embryo over time.

Recently, another *ex ovo* culture in the form of a transparent eggshell was developed using polydimethylsiloxane (PDMS) [224]. This culture system offers superiority over existing *ex ovo* culture systems as it provides transparency, sterility, reusability and biocompatibility. In addition, chicken embryos cultured in this system remained alive for 19 days. Although the transparency of eggshell helps in visualizing the embryo and the vasculature, visibility of organs and vasculature within the embryo decreases over time as the embryo descends slightly into the yolk. This called for the need of an imaging technique which can help in repeatedly observing an embryo cultured in bioengineered eggshell, throughout its incubation period, without making any incision and penetration in the embryo.

Several such non-invasive imaging modalities including MRI, ultrasound, OCT, and micro CT have been used for chicken embryo imaging. Studying vasculature growth is not possible using 7T MRI due to its poor resolution and low hemoglobin contrast [225]. Limited imaging depth of confocal microscopy [226] and OCT [227] makes it difficult to view the embryo at later stages of development. Other imaging techniques like bioluminescence imaging (BLI) [216], intravital imaging [221], laser speckle imaging [228], and high-frequency ultrasound imaging [218] are not widely used for vasculature imaging of chicken embryo as they either need external contrast agent to distinguish the embryonic tissues and organs, have poor imaging resolution or need complicated image processing algorithms to enhance the image quality. Providing the advantages of high ultrasound resolution and good intrinsic optical contrast, photoacoustic imaging seems like a promising choice for chicken embryo imaging.

PAM has been used for vasculature imaging of chicken embryo CAM, for flow speed measurement [229] and for studying the effects of angiogenesis inhibitors

[230]. However, the imaging depth of PAM is limited hence PAT is normally used for whole-body imaging. Recently, Liu et al, performed whole-body imaging of explanted chicken embryos using dual-modality OCT and PAT [220]. Although the images provided structural information of the embryo, image acquisition was performed by explanting the embryos and placing them in paraformaldehyde solution on top of a polymer sensor. This made it impossible to image the same embryo over time.

Here, we have performed dual-wavelength PAT on chicken embryos. For the first time, we have shown how PAT can be employed for imaging various cross-sections of chicken embryo while maintaining its shape and integrity. Initial experiments were performed on naturally growing embryos, wherein small window size and high attenuation of PA waves by the calcium shell prevented visualization of organs and blood vessels. Therefore, imaging was performed on chicken embryos cultured in bioengineered three-dimensional (3D) transparent eggshell which highly mimics the natural eggshell environment. High hemoglobin absorption in visible spectrum of light (400-700 nm) helps achieve high-contrast blood vessel imaging at these wavelengths. Furthermore, in biological tissues, there is relatively decreased scattering and absorption of light of near-infrared (NIR) wavelengths (700-1200 nm). Therefore, greater imaging depth can be achieved with illumination in NIR region as illustrated in Chapter 3. To achieve both high blood contrast and good imaging depth, PAT was performed at wavelengths of 532 and 740 nm. Intrinsic contrast from hemoglobin and eye of the embryo provided cross-sectional images across different depths. Thus, we have shown a label-free method for imaging live chicken embryo cultured in bioengineered eggshells at different developmental stages.

FIGURE 5.1: Schematic of photoacoustic tomography system used for imaging of chicken embryo. M1-M4, mirror; P1-P3, right-angled prism; OD, optical diffuser; R/A/F, receiver/amplifier/filter; PA, photoacoustic; TS1, TS2, translation stage; WT, water tank; UST, ultrasound transducer; SM, stepper motor; RD, rotating disc; DAQ, data acquisition card; LJ, lab jack.

5.2 Materials and Methods

5.2.1 PAT system

Circular scan PAT system described in Chapters 2 and 3 was used for acquiring cross-sectional images of chicken embryo. Schematic of the system with embryo sample is shown in Figure 5.1. Due to ease of availability, 532 nm is extensively used for photoacoustic imaging, therefore, it was chosen for this work as well. For deeper penetration, wavelength in NIR-I region (700-900 nm) have been extensively used in the literature. Here, 740 nm was chosen for the stability of the OPO laser and the higher laser energy output in this wavelength. Maximum fluence per pulse delivered at the surface of the sample was maintained at $\sim 6 \text{ mJ/cm}^2$ for 532 nm and $\sim 5.1 \text{ mJ/cm}^2$ for 740 nm wavelengths. For both the wavelengths, the fluence per pulse was well below the maximum permissible exposure limit, which is 20 mJ/cm^2 for 532 nm and 24 mJ/cm^2 at 740 nm for skin exposure [140].

PA signal generated from the sample was collected using an unfocused single element UST (Olympus NDT, V309-SU), having a central frequency of 5 MHz, a $\sim 70\%$ nominal bandwidth and a 13 mm diameter active area. A stepper motor (Lin Engineering, Silverpak 23C) was used to rotate the UST continuously at a speed of $1.5^\circ/\text{sec}$ to collect time-resolved signals (A-lines) from different angles around the sample. Both the sample and the UST were placed in a water tank to enable better coupling of PA signal by avoiding any air gap between the sample and the transducer. The water tank was kept on a laboratory scissor jack (lab jack) so that it could be easily moved in vertical direction. The acquired PA signal was amplified by ~ 49 dB and filtered using a pulse amplifier (Olympus-NDT, 5072PR). A data acquisition card (DAQ) (GaGe, compuscope 4227) inside a desktop computer was used to record the signal at a sampling rate of 25 MS/s. Data acquisition was synchronized with laser excitation using LabView program. PRR of 10 Hz and scan time of 240 seconds yielded a total of 2400 A-lines in one rotation. Once acquired, 10 consecutive A-lines were averaged into one A-line, thereby converting 2400 A-lines to 240 A-lines. These 240 A-lines were reconstructed using simple delay-and-sum reconstruction algorithm. This reconstruction provided the initial pressure rise distribution (which is related to absorption coefficient of the tissue) of the sample (tissue) in the imaging plane. This system can provide a spatial resolution of $\sim 182 \mu\text{m}$ and an imaging depth of ~ 3 cm in biological tissues [126].

5.2.2 Sample preparation for imaging

5.2.2.1 Phantom experiments

Experiments were first conducted to study the effects of the presence of natural eggshell, and of PDMS on detected PA signal. In these experiments, an absorbing target was irradiated with laser pulses. Twenty PA signals (A-lines) from the target were acquired and averaged. Experiments were repeated 10 times. Following this control experiment, a piece of natural calcium eggshell was placed, first in optical path and later in acoustic path. In both the cases, PA signal was acquired in the

FIGURE 5.2: Photographs of (a) bioengineered eggshell and (b) eggshell fixed inside the holder. (c) Schematic of process used to transfer the fertilized chicken embryo inside the bioengineered eggshell.

same manner as that of control experiment. Later, calcium eggshell was replaced with a piece of PDMS eggshell, and PA signal was acquired in the same manner. Finally, PDMS eggshell was placed in both acoustic and optical path to collect the PA signal from the target. This experiment was repeated for both 532 and 740 nm wavelength excitation. Peak to peak PA signal was calculated from the PA signal A-line by subtracting the minimum value of PA signal from the maximum value of PA signal.

5.2.2.2 Bioengineered eggshell design

A transparent bioengineered eggshell was designed to imitate the geometry of an oversized natural eggshell with a flat top surface. The bioengineered eggshell was prepared from a mixture of PDMS elastomer and a curing agent (Sylgard 184, Dow Corning, Midland, Michigan) in the ratio of 10:1 (wt/wt), using rotation molding at speed ratio of 3:1 via a biaxial bioreactor. A removable transparent lid covered

the top surface of the eggshell to maintain sterility. Minor changes were made to the design of a previously reported bioengineered eggshell [224] to accommodate photoacoustic imaging. Figure 5.2(a) shows a photograph of the bioengineered eggshell used for our experiments. Top narrow portion of bioengineered eggshell was removed as its wider circumference (having a top diameter of 50 mm) aided in increasing the area of illumination during photoacoustic imaging. Furthermore, the eggshell was weighted down by 10 mL of PDMS added to the base to prevent it from floating in the water tank during imaging. An egg holder was designed with CAD software (SOLIDWORKS 2016) and 3D printed (MakerBot Replicator 2) to hold the eggshell stably in the water tank during imaging. Figure 5.2(b) demonstrates the placement of the eggshell into the holder.

5.2.2.3 Chicken embryo preparation

Fertilized chicken eggs (Leghorn x Golden Comet) incubated at 37.9°C and 45% relative humidity (Brinsea Octagon 20 Advance Digital Egg Incubator) were used for the experiments. PAI with excitation at 532 and 740 nm was conducted on both chicken embryos incubated in natural eggshell and bioengineered eggshell, respectively, at 6 days of embryonic life.

In the *in ovo* embryo, PA images of the embryo were acquired at three-time points: before eggshell windowing, following windowing above the airsac, and after removal of the inner membrane. For windowing, a small portion of eggshell on the wider end of the egg was carefully removed to make an opening. Subsequently, the inner membrane was meticulously peeled off exposing the embryo and its vasculature. PA signals collected at different time points were reconstructed to obtain images.

For the *ex ovo* experiments, fertilized eggs were transferred into the bioengineered eggshell for imaging following 55 hours of incubation at 37.9°C and 45% relative humidity in the same incubator as mentioned above. The transfer of fertilized egg into the bioengineered eggshell involved perforating the narrow end of the egg to withdraw 5 mL of albumin, removal of shell above the air sac on the wider end

of the egg, pouring the egg content along with the previously extracted albumin into the bioengineered eggshell, and sealing the bioengineered eggshell with its transparent lid (Figure 5.2(c)). The sealed bioengineered eggshell was placed into the incubator until it was required for imaging. To acquire PA images, chicken embryos at different developmental stages were placed in the water tank and imaged using the PAT system.

For all the experiments, water temperature in the tank was $\sim 38^{\circ}\text{C}$ to provide a viable environment for the embryo. The embryos were immediately replaced in the incubator when they were not being imaged. Abrupt movements were minimized to maintain the viability of the embryos.

5.3 Results and Discussion

Figure 5.3 shows (a), (b) PA signal A-line profiles, and (c), (d) the peak-to-peak PA signal amplitudes from the target. It was observed that no PA signal could be detected/observed when the natural eggshell was placed in either the optical or the acoustic path for both the wavelengths. The peak-to-peak PA signal amplitude in all these cases were less than 5% of the control peak-to-peak PA signal amplitudes (noise level). This is expected, as neither light nor sound can pass through the hard calcium natural eggshell. With PDMS, the PA signal attenuation was observed, but not significantly. Attenuation by $\sim 30\%$ and $\sim 35\%$ was observed on irradiation with 532 nm beam and 740 nm beam, respectively, due to the presence of PDMS eggshell in both the optical and acoustic paths together. Attenuation was quite low when PDMS eggshell blocked either the optical path or the acoustic path. It should also be noted that a slight delay in PA signal was observed when the PDMS shell was placed in path of the acoustic waves. This delay is due to difference in sound speed in the two mediums. Reconstruction algorithms are designed such that this delay does not alter the final reconstructed image.

FIGURE 5.3: a, b, Time resolved photoacoustic signal and c, d, peak to peak photoacoustic signal from the absorbing target with (a, c) 532 nm illumination and (b, d) 740 nm illumination. Ca_light and Ca_sound denote obstruction of optical and acoustic path respectively using natural calcium shell. Similarly, PDMS_light, PDMS_sound, PDMS.both denote obstruction of optical path, acoustic path and both paths, respectively, using PDMS eggshell.

Following phantom experiments, imaging was performed on chicken embryos to further validate the effects of the presence of different eggshells. Figure 5.4 shows (a)-(e) photographs and (f)-(o) photoacoustic images of chicken embryos on their 6th embryonic day of development (EDD). PA images were collected by irradiating the embryos at (Figure 5.4(f)-(j)) 532 and (Figure 5.4(k)-(o)) 740 nm. Initially, PA images of embryo growing naturally were acquired from (Figure 5.4(f),(k)) intact eggshell, (Figure 5.4(g),(l)) windowed eggshell, and (Figure 5.4(h),(m)) eggshell

FIGURE 5.4: Photos of chicken embryo in (a) natural eggshell, (b) natural eggshell with shell removed from top, (c) natural eggshell with shell and outer membrane removed from top, (d) bioengineered eggshell and (e) bioengineered eggshell with lid removed. Photoacoustic image of A-E at 532 and 740 nm are given in f-j and k-o, respectively. Scale bar for f-o is shown in o.

with inner membrane also removed. No distinct features of the embryo were visible in the acquired PA images at both wavelengths. This is due to obstruction (and lack of generation) of PA waves because of the presence of calcium eggshell (as shown earlier).

Figure 5.4(d), (e) show photographs of chicken embryos developing in bioengineered eggshells with (Figure 5.4(d)) lid intact and (Figure 5.4(e)) lid removed. Figure 5.4(i), (n) and (j), (o) are corresponding PA images of Figure 5.4(d), (e), respectively. Analogous to phantom results, attenuation of PA signal due to bioengineered eggshell was significantly less than the natural eggshell. No substantial difference was seen in PA images acquired with and without presence of the lid covering the bioengineered eggshell. However, when bioengineered eggshells covered with lids were placed outside the incubator for longer than 10 minutes, condensation on the internal side of the lid decreased the contrast of acquired PA images (not shown). To minimize condensation, the total scan time was kept within 4 minutes. In Figure 5.4, considerable differences in embryo structures were present

FIGURE 5.5: (a-d) Photos of chicken embryo developing in bioengineered eggshell on embryo development (a) day 4, (b) day 5, (c) day 6 and (d) day 7. (e-h) Magnified images (area shown by dashed lines) and (i-l) photoacoustic images of a-d, respectively. PA images were collected after illumination at 532 nm wavelength. Arrows and numbers in e-h show corresponding structures in i-l, respectively. Scale bar for a-d and e-h are shown in a and e, respectively.

between images collected at 532 and 740 nm. While acquiring PA images, it was necessary that the laser beam illuminated the embryo. However, there was a slight change in alignment of laser beam on changing the wavelength. Therefore, the position of eggshell was changed to ensure that the beam illuminated the embryo. The change in position of eggshell led to change in embryonic position in PA images for different illumination wavelengths. Moreover, the dissimilarity in beam diameter, penetration depth and absorbance of chromophores at different wavelengths resulted in variation of embryonic features visible at different illumination wavelengths. To study this variation further, embryos in different developmental stages were imaged at both the wavelengths separately.

Figure 5.5 and Figure 5.6 show PA images of chicken embryo collected at 532 and 740 nm, respectively. Embryos growing in bioengineered eggshells were imaged

FIGURE 5.6: (a-c) Photos of chicken embryo growing in bioengineered eggshell on (a) day 3, (b) day 4 and (c) day 6 after incubation. Dotted area in a-c is shown in d-f, respectively. g, h, and i are photoacoustic images of a, b and c, respectively, collected after illumination with laser pulses at 740 nm wavelength. (j) Photoacoustic images of c at different cross sections. Scale bar for g-i is shown in g.

from EDD-4 to EDD-7 after illumination at 532 nm. Figure 5.5(a)-(d) show the top view photos of chicken embryos developing in the bioengineered eggshell on different days of development. Multiple embryos were imaged during the study. Figure 5.5(a) and (c) show images of the same embryo on different developmental days, while the rest are of different embryos. Magnified view of the selected region in Figure 5.5(a)-(d) (black dotted square) is shown in Figure 5.5(e)-(h), respectively. Figure 5.5(i)-(l) show the corresponding PA images acquired when the 532 nm beam was aligned to irradiate the area shown in Figure 5.5(e)-(h). Numbers marked in Figure 5.5(e)-(h) show the corresponding regions detected in Figure 5.5(i)-(l). Due to high absorbance of blood at 532 nm, superficial vasculature was clearly visible in the acquired PA images, however, it was difficult to clearly discern the embryonic structures owing to the limited penetration depth of 532 nm.

The photographs of the vasculature in Figure 5.5 vary from the corresponding PA images due to difference in nature of the acquired image. Photographs show the top view of the egg, while the PA images reveal the chromophores present at a particular cross-section. Cross-sectional imaging can help in attaining 3D information of the embryo development, which is difficult to obtain in normal photographs. Dark regions visible in Figure 5.5(h) show the embryonic structures which are not visible in corresponding PA image (Figure 5.5(l)). This is due to the difference in depth at which the embryo was present and at which the PA image was collected. Similarly, some vessels (marked by arrows 3 and 4 in Figure 5.5(h) and (l)) were more clearly visible in the PA images than in the photographs. This is because they were present along the cross-section which was imaged. Moreover, some vessels which were slightly covered by the yolk and not directly visible to the eye, could be seen in the PA images due to the higher absorption coefficient of blood at 532 nm as compared to that of yolk. Although this wavelength greatly aided in studying the development of superficial blood vessels of the embryo, a higher penetration depth was required to study the complete embryonic development.

For imaging at higher penetration depth, embryos developing in bioengineered eggshells were imaged at 740 nm. Figure 5.6 shows top view photos (Figure 5.6(a)-(c)), magnified photos (Figure 5.6(d)-(f)), and photoacoustic images (Figure 5.6(g)-(i)) of chicken embryos at day 3 (Figure 5.6(a), (d), (g)), day 4 (Figure 5.6(b), (e), (h)), and day 6 (Figure 5.6(c), (f), (i)) of development, respectively. In the images, it can be observed that fewer blood vessels were visible at this wavelength, as compared to 532 nm. This may be due to both decreased hemoglobin absorbance as well as a reduced spot size of beam at this wavelength. However, a higher penetration depth of 740 nm compared to 532 nm increased the visibility of embryonic structures, particularly of the eyes, in the acquired PA images. Arrows marked 1, 2 and 3 in Figure 5.6(g)-(j) show the top eye, bottom eye and the heart of the embryo, respectively. The PA images clearly showed gradual development of the eyes over time. Our observations showed that the eyes were barely visible on day 3, however, their contrast greatly increased on days 4 and 6. This was expected

as development of eyes generally takes place after day 3 [215]. Images of different cross-sections were collected by moving the water tank in vertical directions with the help of the lab jack. Figure 5.6(j)(i-vi) show images of six different cross-sections acquired on EDD-6. Precise distance between different cross-sections could not be calculated with the set-up used here. Imaging of multiple cross-sections on EDD-3 and EDD-4 (data not shown) for an extended period of time probably affected the survivability of the embryo due to its removal from the ideal incubator conditions. Consequently, a different embryo was chosen for imaging on EDD-6. Decreasing the scan time by using circular array transducers can help in acquiring full body 3D images of embryo in shorter time duration.

Similar to Figure 5.5, many features of the embryo which were not visible in the photographs could easily be seen in the PA images. Only one eye was distinguishable in the photograph taken from a digital camera, while both eyes could be easily discerned in the PA images. Precisely measuring the displacement of water tank in vertical direction can help in accurately finding the relative depth of different vessels and organs. Furthermore, decreasing the field of view of UST by using a cylindrically focused UST having a narrow profile of detection in the depth direction (vertical direction), can aid in mapping the embryonic structures in 3D. Taking these steps can aid in comparing the embryonic development on different days to quantitatively measure the changes in dimension of blood vessels and organs.

Acquired images in Figure 5.5 and Figure 5.6 demonstrated the capability of PAI to systematically study the growth of chicken embryos. Use of non-ionizing radiations in PAT minimizes the exposure of chicken embryo to harmful ionizing radiations (X-rays used in CT or gamma rays used in PET/SPECT) during imaging. Moreover, the fast acquisition time prevents the need of keeping the embryo outside the incubator for long. These factors aid in maintaining an ideal environment for normal growth of chicken embryo. The laser radiation did not seem to have harmful effects on embryonic development. However, further investigation on a larger sample size may be required to confirm this finding, and to optimize the conditions for PA imaging. Since chicken embryos are vastly studied in developmental and

cancer biology, PAT imaging of chicken embryos in bioengineered eggshells can be used for various applications, that is, to study angiogenesis, effects of genetic manipulations, tumor models, vascular perfusions, etc. Different structural information can be attained by performing PAT at different wavelengths. Additionally, functional imaging can be performed by using multiple wavelengths. Wavelengths beyond 900 nm (e.g., 1064 nm is easily available) can also be employed to increase the penetration (imaging) depth. These wavelengths were not chosen for this work as absorption coefficient of blood is low in these wavelengths. Use of contrast agent with 1064 nm excitation is another possibility for embryo imaging, which can be explored in the future [129]. Different organs or structures of the developing embryo can be targeted using such external contrast agents [132]. Employing UST with higher central frequency can improve the spatial resolution of the system, thus, enabling visualization of smaller vasculature [110]. Furthermore, decreasing the scanning time using multiple ultrasound transducers [38], or lasers with high repetition rate [231, 232] can help in performing real time imaging.

In summary, we have shown the potential of photoacoustic tomography for imaging chicken embryos growing in bioengineered eggshells. High intrinsic contrast was achieved by using wavelengths in visible and NIR-I range. Imaging at 532 nm aided in detecting vascular structure of the developing embryo while the higher penetration depth of 740 nm helped in imaging the embryonic structures (eg, eyes and heart) which had sunk inside the yolk. We have thus reported an application of PAT for imaging the same embryo for consecutive days. This can help in studying embryonic development at different stages.

5.4 Conclusion

Here, we have shown a label-free and high-resolution method of imaging live chicken embryo. Chicken embryos developing in bioengineered eggshells were imaged using photoacoustic tomography at 532 and 740 nm at different developmental stages. Due to high hemoglobin contrast, vasculature development was clearly discernible

at 532 nm. Along with the vasculature, the eyes and the heart were visible at 740 nm. Furthermore, cross-sectional imaging helped in identifying the depths at which different organs and vasculatures were present. By performing PAT on the same chicken embryo developing in bioengineered eggshell, its development over time can be easily monitored. Moreover, using this method, effects of various drugs, genetic modifications or chemicals on embryonic development can also be examined. Therefore, we believe photoacoustic tomography can be a promising option for imaging the development of chicken embryos over time.

Chapter 6

Conclusion and future prospects

6.1 Conclusion

In this dissertation, improvement and optimization of photoacoustic imaging systems and their application for biomedical imaging have been presented. Methods of improving data acquisition time, imaging depth, and imaging resolution of different types of photoacoustic imaging system have been shown, and a novel application of an optimized circular scan single element transducer-based photoacoustic tomography system for chicken embryo imaging has been proposed. The aim of this thesis was to compare various aspects of photoacoustic imaging system, enhance the systems for different applications, and illustrate biomedical applications of the system.

Different scanning methods of single element-based circular scan photoacoustic tomography system were compared to demonstrate the improvement in data acquisition time by using continuous scanning instead of stop-and-go scanning method. Single element transducer provides the advantages of being highly sensitive, cheap, and easily available, however, as compared to array-based transducers, SET-based PAT systems have a longer data acquisition time. We showed that for small animal imaging, continuous scans can reduce the scanning time of SET-based PAT

systems without compromising the image quality. A 2-4 folds and a 7-12 folds improvement in scan time was demonstrated on using traditional Nd:YAG laser based PAT system and PLD-based PAT system, respectively. The decrease in acquisition time also led to a corresponding decrease in number of laser pulses irradiating the sample. Moreover, no substantial decrease in image quality parameters like signal to noise ratio, resolution, or spatial accuracy countered this speed improvement.

Following enhancement of data acquisition time, the dependence of imaging depth on wavelength of optical illumination during photoacoustic imaging, was studied. Monte Carlo simulations and photoacoustic experiments were performed to compare the photoacoustic signals at different depths after irradiating breast tissue at 532-, 800-, and 1064-nm wavelengths. Simulations and experiments were designed to mimic vasculature imaging and imaging of SLN using external contrast agent. We showed that when same illumination energy at all wavelengths is used, wavelengths in NIR-I window have highest penetration due to decreased scattering and absorption. However, 1064 nm (NIR-II window) has the advantage of having higher maximum permissible energy for irradiating the sample. Therefore, when the fluence at which different wavelengths irradiate the sample is proportional to the MPE at that wavelength, 1064 nm had higher penetration. These results were validated with experiments conducted on circular scan photoacoustic tomography system and acoustic resolution photoacoustic microscopy experiments. This study can help in selecting and optimizing the wavelength to be use for photoacoustic imaging.

Moving further, we proposed application of deep learning algorithms to improve the image quality of acoustic resolution photoacoustic microscopy images. We showed how the resolution of AR-PAM images outside the focal zone can be improved after evaluating the images using a trained fully dense U-Net network. B-scans mimicking those of an AR-PAM system were simulated to train the FD-UNet model. Simulated images tested on the model showed mean square error and structural similarity index of 0.7 and 99.88%, respectively, between the CNN-based and the ground truth images, thus validating that the model had been trained.

Testing the model on experimental data indicated that using such models can help in maintaining almost similar resolution across the entire imaging depth. Moreover, we also demonstrated that apart from improving the resolutions, such models can also improve the image quality by enhancing the signal strength, decreasing the background noise, and removing background skin signal.

After optimizing the imaging system, we explored the application of such systems for studying the development of chicken embryos. Chicken embryos were cultured in bioengineered eggshells which provided it with a safe and viable environment to grow and develop. Circular scan photoacoustic tomography system was employed to perform cross sectional imaging of these embryos. High resolution, label-free imaging of the embryos at multiple developmental stages was performed after irradiating them at 532- and 740-nm wavelengths. It was demonstrated how photoacoustic tomography can be used to visualize the blood vessels and the developing organs like eyes and heart of the chicken embryo. Images of the embryo were acquired across various cross-sections, exhibiting the ability of such imaging method to determine the depth at which different vessels are present during each stage. Hence, we showed that photoacoustic imaging has the capability of studying embryonic development.

6.2 Future directions

The last two decades has seen an exponential growth in research and advancement of photoacoustic imaging for biomedical applications. However, for any imaging modality, efforts are constantly made to modify and improve the imaging systems such that it can be utilized to its full extent to provide images that can help in clinical and pre-clinical applications, while being safe and convenient to use. Aim of most imaging systems is to provide real-time images with high contrast, high resolution, at higher depths, and at lower costs. Moreover, the system design should be such that they are portable, versatile, and easy to use. Although the

present photoacoustic imaging systems provide many of these advantages, there is still room for improvement.

For any PAI application, selecting the wavelength for irradiating the sample is crucial. The wavelength should be chosen such that the target tissue/contrast has high absorbance in that range, while the surrounding tissues has low absorbance as well as scattering. Furthermore, a higher maximum permissible energy may result in deeper penetration of light. Availability of low-cost lasers also influences the choice of wavelength to be used. Even higher imaging depths may be obtained on using wavelengths beyond 1064 nm. Fluence at different depths in various body tissues should be compared when irradiated with such high wavelengths as well. This will help in optimizing the wavelength for different biomedical application. Development of food and drug authority (FDA) approved contrast agents having absorbance at the optimized wavelengths can further improve the acquired images.

Capacity of deep learning algorithms to improve PAI images is far beyond what has been proposed till now. The proposed method can be extended to include improvement in out-of-focus resolution of AR-PAM images acquired using ultrasound transducers having different central frequencies and/or focal lengths. Also, including light propagation pattern can help in mapping the absorbance more accurately. This may be used to perform functional imaging as well. Adding skin signal and more structures having different dimensions may further improve the images predicted by the model. Apart from AR-PAM, DL can also help in improving resolution, signal to noise ratio, and image quality of images acquired using other PA systems. Various DL algorithms should be further explored to improve image quality, reduce scan time, and decrease the cost of PA imaging system.

Chicken embryo has helped in some major biological breakthroughs and still remains to be an important model for pre-clinical biological studies. We have shown the potential of using photoacoustic tomography to acquire images of development in same chicken embryo at multiple developmental stages. We propose to use this

model to study effects of various drugs and genetic modifications on embryonic development. Using a sophisticated stage in the set-up to determine the precise depth of organs and vessels and using external contrast agent to tissues and organs which have weak intrinsic contrast can improve the system further. PAM can be used instead of PAT might to get high resolution three dimensional images. We believe that this model can be efficient for cardiovascular and developmental studies.

Various other methods for advancement of PA imaging systems also need to be analyzed. Developing a real-time, low-cost, portable AR-OR-PAM system can broaden the scope of applications of PAM. Using NIR-II wavelengths with external contrast agents can provide improved imaging depth. This method can help in visualizing tumor growth at high imaging resolution. Additionally, by using multiple wavelengths in this system, functional imaging can be performed as well. Further, improving the resolution of OR-PAM beyond the optical diffraction limit ($\sim 0.5\lambda/NA$ where λ is the optical wavelength and NA is numerical aperture of optical objective) can help in label-free imaging of nanoparticles, single cells, and even cellular organelles. We propose to use photonic nanojet generated by silica microspheres [105] for this purpose. This can be a simple, cost-effective, and non-destructive way for super-resolution imaging.

In summary, photoacoustic imaging has shown promising potential for use in healthcare sector. Nevertheless, there is still room for refinement and optimization of these imaging systems. Moreover, there exist multiple biomedical applications of PA yet to be explored. We intend to further modify the system and make them versatile so that the imaging systems can be availed for more pre-clinical and clinical applications.

List of Publications

Journal Articles⁵

1. D. Das, **A. Sharma**, P. Rajendran, M. Pramanik, Another decade of photoacoustic imaging, *Physics in Medicine and Biology* (2021) (In Press).
2. **A. Sharma**, M. Pramanik, Convolutional neural network for resolution enhancement and noise reduction in acoustic resolution photoacoustic microscopy, *Biomedical Optics Express* 11(12), 6826-6839 (2020)
3. **A. Sharma***, N. Ishak*, T. Swee-Hin, M. Pramanik, High resolution, label-free photoacoustic imaging of live chicken embryo developing in bioengineered eggshell, *Journal of Biophotonics* 13(4) (2020) e201960108.
4. **A. Sharma**, Srishti, V. Periyasamy, M. Pramanik, Photoacoustic imaging depth comparison at 532-, 800-, and 1064-nm wavelengths: Monte Carlo simulation and experimental validation, *Journal of biomedical optics* 24(12) (2019) 121904
5. **A. Sharma**, S.K. Kalva, M. Pramanik, A comparative study of continuous versus stop-and-go scanning in circular scanning photoacoustic tomography, *IEEE Journal of Selected Topics in Quantum Electronics* 25(1) (2019) 7100409.
6. V. Periyasamy, N. Das, **A. Sharma**, M. Pramanik, 1064 nm acoustic resolution photoacoustic microscopy, *Journal of Biophotonics* 12(5) (2019) e201800357.

⁵the superscript * indicates joint first authors

7. M. Mozaffarzadeh*, M.H.H. Varnosfaderani*, **A. Sharma***, M. Pramanik, N.D. Jong, M.D. Verweij, Enhanced contrast acoustic-resolution photoacoustic microscopy using double-stage delay-multiply-and-sum beamformer for vasculature imaging, *Journal of Biophotonics* 12(11) (2019) e201900133.
8. Y. Jiang, P.K. Upputuri, C. Xie, Z. Zeng, **A. Sharma**, X. Zhen, J. Li, J. Huang, M. Pramanik, K. Pu, Metabolizable Semiconducting Polymer Nanoparticles for Second Near-Infrared Photoacoustic Imaging, *Advanced Materials* 31(11) (2019) 1808166.
9. M. Moothanchery, **A. Sharma**, M. Pramanik, Switchable Acoustic and Optical Resolution Photoacoustic Microscopy for *in vivo* small-animal blood vasculature imaging, *Journal of Visualized Experiments* (124) (2017) e55810.

Conference Proceedings

1. **A. Sharma**, S.K. Kalva, M. Pramanik, Comparison of continuous and stop-and-go scanning techniques in photoacoustic tomography, *Proc SPIE*, 2018, p. 104943L.
2. M. Moothanchery, **A. Sharma**, V. Periyasamy, M. Pramanik, High resolution and deep tissue imaging using a near infrared acoustic resolution photoacoustic microscopy, *Proc SPIE*, 2018, p. 104945R.
3. **A. Sharma**, N. Ishak, T. Swee-Hin, C. Cheung, M. Pramanik, Photoacoustic imaging of live chicken embryo at multiple developmental stages, *Proc SPIE*, 2020, p. 1124030.
4. **A. Sharma**, V. Periyasamy, M. Pramanik, Monte Carlo simulations and photoacoustic experiments to compare imaging depth at 532 nm, 800 nm, and 1064 nm, *Proc SPIE*, 2020, p. 1124043.

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