Dual modality ultrasound - photoacoustic clinical imaging system and its applications

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**NANYANG
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DUAL MODALITY ULTRASOUND - PHOTOACOUSTIC CLINICAL IMAGING SYSTEM AND ITS APPLICATIONS

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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**DUAL MODALITY ULTRASOUND -
PHOTOACOUSTIC CLINICAL IMAGING SYSTEM
AND ITS APPLICATIONS**

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Abstract

Photoacoustic (PA) imaging is a rapidly growing hybrid biomedical imaging modality. Translating it to the clinic still remains a challenge which needs to be addressed. Using a clinical ultrasound imaging system if one can do dual modal ultrasound as well as photoacoustic imaging, it will be a big step towards clinical translation. Such system will provide the flexibility of combining with a variety of lasers and transducers to image various different body parts. By combining it with a high repetition rate pulsed laser diode, high frame rate PA imaging can be achieved. The high frame rate imaging is highly useful for real-time photoacoustic imaging.

Energy from pulsed laser diode is low, and not all applications require high frame rate imaging. Therefore, a frequency doubled Nd:YAG laser pumping an optical parametric oscillator was also combined with the clinical ultrasound system for handheld dual modal ultrasound and photoacoustic imaging. A bifurcated optical fiber used for light illumination was integrated with the ultrasound probe. The imaging angle, the distance between fiber and tissue etc. was optimized using Monte Carlo simulations to finalize the handheld imaging probe holder design.

The handheld probe was used for two different pre-clinical applications: sentinel lymph node imaging, and urinary bladder imaging. Firstly, sentinel lymph nodes in rats was imaged with methylene blue (675 nm) and indocyanine green (795 nm) as contrast agent. Non-invasive, real time needle tracking was also performed. Imaging depth similar to human imaging scenario was demonstrated. Furthermore, combined ultrasound and PA imaging of rat urinary bladder was demonstrated with the handheld probe. Structural and functional bladder imaging was shown. Also, the feasibility of PA imaging for a common bladder disorder, vesicoureteral reflux was studied using urinary

tract mimicking phantoms. Additionally, non-invasive clearance study of gold nanorods from circulation was done by monitoring the accumulation of the gold nanorods in the bladder.

Contrast agents are very important for improving photoacoustic signal strength and having multiple FDA approved contrast agent will make clinical translation more feasible. Two different contrast agents were explored in this work. With the development of combined dual modal imaging, dual modal contrast agents are needed. A dual modal organic dyes containing nitrogen microbubbles ($<7\ \mu\text{m}$) generated from a flow focusing junction based microfluidic device. Here, the organic dyes (methylene blue and black ink) will provide the photoacoustic contrast and the nitrogen microbubbles will offer the ultrasound contrast. The efficiency of the contrast agent was initially tested in tube phantoms and later in *in vivo* studies. Another contrast agent explored is a potential theranostics agent, near-infrared light-sensitive liposomes coated with gold nanostars (AuNSs) for both PA imaging and drug release. We explored the use of AuNSs-coated liposomes as contrast agents for PA imaging with tissue phantom experiments.

Table of contents

Acknowledgement	3
Abstract.....	5
Table of contents	7
List of figures.....	10
List of tables.....	11
Abbreviations	12
Chapter 1	15
Introduction to clinical photoacoustic imaging.....	15
1.1 Introduction to clinical imaging.....	15
1.2 Photoacoustic imaging	21
1.3 Challenges associated with clinical translation.....	26
1.4 Structure of thesis	27
Chapter 2	29
High frame rate photoacoustic imaging.....	29
2.1 Introduction.....	29
2.2 Methods	32
2.2.1 PLD-PAT imaging system.....	32
2.2.2 OPO-PAT imaging system	33
2.2.3 PA data acquisition and image formation	34
2.2.4 Ultrasound imaging.....	35
2.2.5 Phantom experiments.....	36
2.2.6 Laser Safety	37
2.3 Results and Discussion	38
2.4 Conclusion	43
Chapter 3	44
Handheld probe for dual modal US-PA imaging.....	44
3.1 Introduction.....	44
3.2 Methods	47
3.2.1 Monte Carlo simulation for designing the probe	47
3.2.2 Photoacoustic and optical spectrum of methylene blue	51
3.2.3 Design of the fiber bundle holder	51
3.2.4 PAT imaging system.....	52
3.2.5 Resolution characterization.....	53
3.2.6 Phantom experiments.....	55

3.3 Results and discussion	56
3.4 Conclusion	63
Chapter 4	65
Applications of clinical PA imaging	65
4.1 Non-invasive sentinel lymph node mapping and needle guidance	65
4.1.1 Introduction.....	65
4.1.2 Materials and Methods.....	68
4.1.2.1 Photoacoustic and optical absorbance spectrum of the dyes used	68
4.1.2.2 Clinical handheld photoacoustic imaging system	69
4.1.2.3 Animal preparation	69
4.1.2.4 Sentinel lymph node imaging in rats.....	70
4.1.2.5 Needle tracking	72
4.1.3 Results and Discussion	72
4.1.4 Conclusion	81
4.2 Rat urinary bladder and its applications.....	82
4.2.1 Introduction.....	82
4.2.2 Materials and Methods.....	85
4.2.2.1 US-PA imaging system.....	85
4.2.2.2 Animal preparation	86
4.2.2.3 Structural imaging of bladder	87
4.2.2.4 Flow imaging	87
4.2.2.5 Clearance imaging	88
4.2.2.6 Laser safety	88
4.2.3. Results and discussions.....	89
4.2.4. Conclusion	95
Chapter 5	96
Contrast agents for PA imaging	96
5.1 Dual modal ultrasound/photoacoustic contrast agent	96
5.1.1 Introduction.....	96
5.1.2 Methods	99
5.1.2.1 Microbubbles generation in methylene blue and black ink.....	99
5.1.2.2 Dual modal US-PA imaging system	101
5.1.2.3 Phantom imaging	101
5.1.2.4 Animal preparation and imaging	102
5.1.2.5 Laser safety	103
5.1.3 Results and Discussion	103
5.1.4 Conclusion	111
5.2. Near Infrared light-responsive liposomal contrast agent.....	113

5.2.1 Introduction.....	113
5.2.2 Materials and methods	115
5.2.2.1 Synthesis of Liposomes coated with gold nanostars	115
5.2.2.2 Characterization of liposomes loaded with AuNSs	116
5.2.2.3 Cell culture.....	117
5.2.2.4 Photoacoustic Spectrum of liposomes loaded with AuNSs	117
5.2.2.5 Photoacoustic signal from liposome and animal blood.....	118
5.2.2.6 Deep-tissue photoacoustic imaging experiment.....	118
5.2.2.7 Photoacoustic imaging of melanoma cells.....	119
5.2.2.8 Drug Release Studies	119
5.2.2.9 Qualitative Analysis of <i>in vitro</i> Calcein Release	120
5.2.2.10 Cytotoxicity Assay.....	121
5.2.3. Results and Discussion	121
5.2.4 Conclusion	129
6. Conclusion and future directions	130
6.1 Conclusion	130
6.2 Future directions	132
Appendix: Modification in the Monte Carlo	134
Publications	136
References	138

List of figures

Figure 1: Various clinical imaging system used for diagnosis and treatment.	16
Figure 2: Optical absorption spectrum of the most commonly occurring chromophores in the body.....	25
Figure 3: Schematic of the PLD-PAT ultrasound setup	33
Figure 4: Ultrasound B-mode image.....	39
Figure 5: Flow imaging with PLD-PAT system at 3500 fps	40
Figure 6: Flow imaging with PLD-PAT system at 3500 fps	40
Figure 7: Flow imaging with OPO-PAT system.....	41
Figure 8: Monte Carlo simulation geometry and fiber based light illumination design	49
Figure 9: Schematic representation of the imaging system and components..	54
Figure 10: Axial resolution characterized at different depths.....	54
Figure 11: Optical and PA spectrum of methylene blue.....	56
Figure 12: Simulation results.	58
Figure 13: Experimental results with SLN phantoms.....	60
Figure 14: Schematic representation of the PA imaging system for SLN imaging.....	71
Figure 15: In-vitro optical and photoacoustic spectrum.....	73
Figure 16: In-vivo SLN mapping in rats.....	75
Figure 17: In-vivo SLN spectroscopic imaging.....	77
Figure 18: In-vivo PA spectrum.	77
Figure 19: In-vivo SLN depth imaging.....	79
Figure 20: Non-invasive needle tracking.....	80
Figure 21: Schematic of the US-PA imaging system for bladder imaging.....	86
Figure 22: Structural imaging of urinary bladder	90
Figure 23: Urinary bladder depth imaging.....	92
Figure 24: Vesicoureteral phantom imaging.....	93
Figure 25: Nanoparticle clearance imaging.	94
Figure 26: Schematic of the flow-focusing junction experimental set-u.....	100
Figure 27: Schematic representation of the PA imaging system.	101
Figure 28: Size distributions of microbubbles.	104
Figure 29: Schematic representation of the PA imaging system	106
Figure 30: In vivo urinary bladder images with microbubbles.....	108
Figure 31: Characterization profile of AuNSs coated liposomes	122
Figure 32: PA spectrum and depth imaging.	123
Figure 33: PA imaging of melanoma cells.	125
Figure 34: Percentage of Drug Release.	125
Figure 35: In vitro Release of Calcein	127
Figure 36: Cell viability study	129
Figure 37: Flow chart of Monte Carlo with embedded sphere (MCES).....	135

List of tables

Table 1: Comparison of different clinical imaging modalities	20
Table 2: Comparison of the flow rates as controlled from the syringe pump and measured from the PA images from the PLD-PAT system.....	42
Table 3: Optical properties of various elements used in the MCES at 675 nm wavelength.	47
Table 4: Comparison of signal to noise ratios (SNR) of US and PA signals for the various samples in tube phantom.....	109
Table 5: Values of signal to noise ratios (SNR) of US and PA signals from the in vivo animal images (average of both sagittal and transverse planes).....	110

Abbreviations

ADC	Analog-to-digital convertor
ALND	Axillary lymph node dissections
ANSI	American National Standards Institute
AR	Acoustic resolution
AuNPs	Gold nanoparticles
AuNS	Gold nanostar
BF	Beam formed
BI	Black ink
CL	Cylindrical lens
CT	Computed tomography
CTC	Circulating tumor cells
DOT	Diffuse optical tomography
FDA	Food and drug administration
FDG	Fludeoxyglucose
fMRI	Functional magnetic resonance imaging
FNAB	Fine needle aspiration biopsy
FPD	Fiber-to-probe distance

Fps	Frames per second
FTD	Fiber-to-tissue distance
FWHM	Full width at half maximum.
GIT	Gastro intestinal tract
ICG	Indocyanine green
LDPE	low density polyethylene
LDU	Laser driver unit
MB	Methylene blue
MCML	Monte Carlo simulation for light propagation in multi-layered tissue
MPE	maximum permissible exposure
MRI	Magnetic resonance imaging
NA	Numerical aperture
NIR	Near-infrared
NIRS	Near-infrared spectroscopy
OCT	Optical coherence tomography
OF	Optical fiber
OPO	Optical parametric oscillator
OR	Optical resolution
PA	Photoacoustic

PAI	Photoacoustic imaging
PAM	Photoacoustic microscopy
PAT	Photoacoustic tomography
PA-US	Photoacoustic ultrasound imaging system
PAUSFI	Photoacoustic ultrasound fluorescence imaging
PET	Positron emission tomography
PL	Plain liposomes
PLD	Pulsed laser diode
RF	Radio frequency
ROI	Region of interest
SC	Scan converted
SLN	Sentinel lymph node
SLNB	Sentinel lymph node biopsy
SNR	Signal-to-noise ratio
TTL	Transistor-transistor logic
UB	Urinary bladder
US	Ultrasound
UST	Ultrasound transducer
VUR	Vesicoureteral reflux

Chapter 1

Introduction to clinical photoacoustic imaging

1.1 Introduction to clinical imaging

Over the years, there has been a steep increase in the occurrence of diseases like cancer, diabetes, heart diseases etc., and the number of deaths related to it is expected to increase in the next few years [1]. Some of these diseases can be very fatal if not diagnosed and treated at the right time [2]. One of the major problem associated with these diseases is that they can remain asymptomatic and hence left undiagnosed for a long period of time. Screening, diagnosing and staging of these diseases is very crucial as it will determine the prognosis of the patient and help the doctors in deciding on the most effective strategy for treatment. Diagnosis is possible by using different imaging techniques or by testing different parameters of blood, urine sample, etc. obtained from the patient. For cancer diagnosis, a histopathological study following biopsy is used as confirmatory study. There are a wide range of imaging modalities available to aid the physicians in the process of diagnosis. Some of the most commonly used imaging modalities are X-Ray projection imaging, X-Ray computed tomography (CT), Magnetic resonance imaging (MRI), Positron emission tomography (PET), ultrasound (US), endoscopy etc. Fig. 1 shows the commonly used clinical imaging systems. There are limitations associated with each of the imaging modality, therefore, there is a constant need for new imaging modality.

Figure 1: Various clinical imaging system used for diagnosis and treatment in the hospitals.

X-rays was discovered by W. Rontgen in 1895. In X-ray imaging electromagnetic radiation in the wavelength range of 0.01 to 10 nm and energy range of 100 eV to 100 KeV is used. The different tissues in the body absorb different amount of radiations, the bone absorbs the most and appears white, whereas soft tissues absorbs very minimally and appears gray, and air the least and looks black in X-ray image. To image soft tissues better, external contrast agents are also used [3]. X-Rays are mostly used for imaging hard tissue like bone to identify fracture, cracks, etc. Additionally, X-rays are also used for screening lung related disorders like tuberculosis, pneumonia, lung cancer etc. X-ray mammography, is one of the most commonly used technique for large scale screening of breast cancer in women [4]. However, in case of dense breast tissue, it is not very effective. Fluoroscopy also uses continuous, real-time X-ray imaging with injection of contrast. It is most commonly used for imaging moving objects like stent placement, heart pumping action etc. Planner X-ray imaging does not provide 3

dimensional information. CT overcomes this by imaging the cross-section of a target with multiple projection angle and then by using a reconstruction technique. Taking image of multiple cross-sections, a full 3D volumetric image of the patient is constructed. The major drawback of using X-ray imaging is the use of ionizing radiation. Although, the dosage is maintained within safety limits, repeated exposure might cause damage to the DNA, which leads to increased risk of cancer in patients. Especially with fluoroscopy and CT the radiation dose that the patient gets exposed to is relatively higher than conventional X-ray imaging.

MRI is an imaging technique which provides information on the anatomy of the body [5]. Unlike X-rays, it does not use any ionizing radiation for imaging but uses magnetic field which is absorbed by specific atomic nuclei (mostly hydrogen atom). A magnetic field of 1.5 T is usually used for clinical imaging. The hydrogen atoms are easily available in the body especially in water and fat. After excitation, the return to equilibrium state happens by two different methods in tissues: T1 (spin-lattice) and T2 (spin-spin) relaxation which provides information from different types of tissues. MRI is commonly used for imaging tumors in the body with high sensitivity [6, 7]. It is used for confirmatory testing of breast tumors especially when the density of the breast tissue is very high [8-10]. MRI is also used for neuroimaging, cardiovascular imaging, gastrointestinal tract, etc. Apart from anatomical information MRI can provide functional information as well. Functional MRI (fMRI) gives information on the changes associated with blood flow to measure brain activity [11, 12]. Although it is a safe imaging modality, it is not the most patient friendly imaging technique. The patient has to lie down in a tube-like structure which makes it unsuitable for claustrophobic patients. However, recently open MRI with very low magnetic field is available for such patients, but it will not be as effective as the closed MRI. Another disadvantage is

that it is not suitable for patients having metallic implants in their body. MRI is also very expensive, which makes it less affordable.

PET is a nuclear medicine functional imaging technique which provides metabolic information about the body and requires radionuclides as contrast agent to be injected in the body [13]. The radionuclides used in PET are typically ones with very short half-lives (Carbon-11, fluorine-18, zirconium-89) ranging from few minutes to few hours, which are incorporated in molecules such as glucose, ammonia, water, etc. Most commonly used active molecule is fludeoxyglucose (FDG), an analogue of glucose which is usually labelled with fluorine-18. The radioisotope emits positron (anti electron) upon positron emission decay. Shortly after positrons are created it comes to interact with an electron leading to annihilation process which leads to the generation of two gamma photons (each with 511 keV) travelling in opposite direction. These gamma photons are detected by the coincidence detector and later images are reconstructed. Rarely PET is used as a standalone imaging system, it is combined with complimentary imaging techniques like CT or MRI which obtains structural imaging and PET obtains the metabolic information. PET is majorly used in cancer imaging. It also finds applications for neuroimaging, cardiovascular imaging, etc. [5, 14]. The major disadvantage is that as the production of the short half-life radionuclides requires cyclotron, which is very expensive. Also preparing the combination of the radioisotope labelled active compound is difficult. Very few hospitals are equipped to maintain them, which further increases the cost.

US is a non-invasive imaging modality, which uses sound waves in ultra-high frequencies above the audible range (1-20 MHz) [15]. US imaging operates on the principle that when US waves are pulsed into tissues using a transducer, the tissue echoes the sound wave back. Different tissues reflect the sound waves to different

degrees. The image is based on the amount of time the echo takes to reach the transducer once the sound is pulsed and also by the strength of the echo. The transducers act as pulse receivers. After receiving, the data is reconstructed to form images that are displayed in real-time on the monitor. B-mode two dimensional images are formed based on the acoustic impedance from the tissues [16]. Blood flow can be imaged in ultrasound in the form of Doppler imaging [17]. US can be used for imaging soft tissues only. US is used for imaging in gynecology and obstetrics. It is also used for imaging the abdominal region, blood vessels, etc. [18]. To improve ultrasound contrast microbubbles can be used [19, 20]. The major advantages of US imaging are it is non-invasive, easily portable, with high penetration depth, high resolution. Some of the disadvantages of US imaging include operator dependency, applicable to only soft tissue imaging (bone imaging or bone penetration of sound waves are limited), poor contrast, and use of acoustic coupling medium.

Endoscopy is an invasive imaging technique used to image the internal organs and cavities of the body. In this method an endoscope is inserted into the body, there is a long optical fiber for light transmission with light source located outside the body. A camera is used to transmit the image to the monitor. Endoscopy is used for imaging the gastrointestinal tract (GIT) most commonly. Images of the internal structure of the different parts of the GIT and can be used to image the tumors present there [21]. The major disadvantage of this technique is that it is invasive which causes a lot of discomfort to the patients. Recently, capsule endoscopy is being developed and used which overcomes the problem of invasiveness to some extent [22]. Also, its application is restricted to very few parts of the body. Additionally, the resolution offered by the technique is not great.

Table 1: Comparison of different clinical imaging modalities

	US	X-ray	CT	MRI	PET	Endoscopy
Access	Small windows adequate	2 sides needed	Circumferential around body	Circumferential around body	Circumferential around body	Inside the body
Spatial Resolution	Frequency and axially dependent; 3 to 0.3 mm	~ 1 mm	~ 1 mm	~ 1 mm	~1 mm	~100 μ m
Penetration	Frequency dependent; 3 to 25 cm	Excellent	Excellent	Excellent	Excellent	Poor
Safety	Very good	Ionising radiation	Ionising radiation	Very good	Radioactive contrast	Invasive
Speed	100 frames per second	Minutes	Half-minute to minutes	Minutes	Seconds to minutes	Real-time
Cost	\$	\$	\$\$\$\$	\$\$\$\$\$\$\$\$	\$\$\$\$\$\$\$\$	\$\$

Some of the other **optical imaging** techniques includes diffuse optical tomography (DOT), near-infrared spectroscopy (NIRS), and optical coherence tomography (OCT), optical microscopy etc. DOT is a non-invasive imaging technique which uses multiple-scattered photons to form images at much deeper tissue depth. It is used for soft tissue imaging majorly. It can be used for measuring total hemoglobin concentration, blood oxygen saturation, etc. DOT can be used for applications including breast cancer imaging, brain functional imaging etc. [23]. NIRS is a spectroscopic method which uses the electromagnetic spectrum in the NIR region. It uses the principle of molecular overtone and combination vibrations. The major applications include pulse oximetry and functional neuroimaging [24]. OCT is an imaging technique that is based on low coherence interferometry. It typically uses light in the NIR region. It can be used for applications like eye imaging, cardiology, etc. [25]. The major limitation of optical imaging techniques is imaging depth, poor imaging resolution at depth, etc. The transport inside biological tissue is characterized by multiple scattering and absorption. Therefore, most high resolution optical imaging (like OCT, optical microscopy etc.) are

limited to few millimeter of imaging depth. DOT uses diffused photons for imaging, thus can image deeper, but with poor resolution. Deep tissue imaging with high resolution is still a challenge in optical imaging.

The currently available clinical imaging systems have many limitations. Therefore, there is a necessity for better imaging modality which is safe, with high resolution and contrast, portable, economical, with greater depth of penetration. Photoacoustic (PA) imaging is a fast growing hybrid biomedical imaging technique which is being explored for different clinical applications.

1.2 Photoacoustic imaging

Photoacoustic imaging (PAI) is a hybrid, non-invasive imaging modality, combining both optical and ultrasound imaging, rapidly gaining importance in the field of biomedical imaging [26]. The PA effect was initially observed by A.G. Bell in the year 1880 [27]. In PAI a short laser pulse irradiates the sample (*e.g.*, biological tissues), leading to absorption of light energy to increase the local temperature (in the order of a few milli degrees), resulting in thermoelastic expansion and release of pressure waves known as photoacoustic (PA) waves. Ultrasound detectors acquire these PA waves outside the sample boundary [28]. Reconstruction techniques are used to form the optical absorption map of the inside of the object [29, 30].

PA can be described through wave equation, which is given by

$$(\nabla^2 - v_s^{-2} \partial^2 / \partial t^2) p(\vec{r}, t) = -(\beta / C_p) \partial H(\vec{r}, t) / \partial t$$

v_s refers to acoustic speed, $p(\vec{r}, t)$ refers to the acoustic pressure at location $\vec{r}$ and time t , β refers to the thermal expansion coefficient, C_p refers to the specific heat constant at constant pressure, and H denotes the heating function which can be described as the thermal energy converted per unit volume and per unit time. The left-hand side of this

equation describes the wave propagation, whereas the right-hand side represents the source term.

PAI has several advantages including deeper penetration depth, good spatial resolution, and high soft tissue contrast. It combines the high resolution of pure ultrasound imaging, and high contrast of pure optical imaging into a single modality [31]. Ultrasound scattering is two to three orders of magnitude less than the optical scattering in biological tissues, making photoacoustic imaging overcome the fundamental depth limitations of existing pure optical imaging. It is also free of speckle artifacts and relatively low cost. PA can be used for functional imaging as PA is sensitive to the concentration and oxygenation of hemoglobin in blood [32]. Another major advantage of PA imaging is that it can be easily combined with other imaging modalities like US, fluorescence, OCT, MRI etc. to form multimodal imaging systems. PAI has several potential clinical applications, such as breast cancer imaging, brain imaging, sentinel lymph node (SLN) imaging, temperature monitoring, circulating tumor cell (CTC) imaging, urinary bladder imaging, imaging of neoangiogenesis, GIT imaging, etc. [33-42].

The major advantage of PAI is that multiscale imaging capability. It can image from organelles to organs and systems with consistent contrast [43]. There are different embodiment of PA imaging systems available which can be used for imaging at various scales, namely, photoacoustic microscopy (PAM) for single cells and very small structures, photoacoustic tomography (PAT) for small organs and systems. PA nanoscopy is also being developed which can be potentially used for imaging ultrafine structures like cell organelles. The PAM can be further classified into acoustic resolution (AR) and optical resolution (OR) PAM systems. The resolution for ARPAM

is approximately 50 μm and ORPAM is around 1-5 μm [44, 45]. The major drawback with the PAM system is that it has very limited depth of penetration ($\sim 1\text{ cm}$). Currently, a lot of research is happening on PA nanoscopy systems which can provide a resolution of less than 100 nm [46]. With such systems, imaging the internal structure of cells will become possible, however, with a very shallow imaging depth (only within few hundreds micron). For deep tissue imaging PAT systems can be used. PAT can be performed with a single element or multiple transducers through circular scanning. Using PAT systems bigger structures like small organs (SLN, urinary bladder, small animal brain) can be visualised. The resolution of these systems will be around 100-200 μm (depending on transducer frequency being used). Array based PAT systems can also be used for imaging bigger structures with a resolution of $\sim 200\text{ }\mu\text{m}$ [33, 47]. The depth of penetration of PAT systems is usually 4 to 5 cm [37]. Photoacoustic endoscopy is also being explored for imaging internal organ and structures [48, 49].

For clinical imaging, PA imaging was combined with the clinical ultrasound systems to perform dual modal ultrasound-photoacoustic imaging. There are different clinical imaging systems. One of the first system was the Philips iU22 system, which was used for small animal sentinel lymph node imaging. The series of programmable US systems by Verasonics, USA, are among the most advanced research systems. It has been tested for various applications by modifications using Matlab-based software. But, these systems are research based systems and cannot be modified by the end user very easily. Additionally, since they are not commercially used clinical systems they would require FDA approval for clinical translation. The VevoLAZR (FujiFilm VisualSonics, Canada), Nexus 128 (Endra Life Science, USA), and MSOT Acuity (iTera Medical, Germany) are provide combined ultrasound and photoacoustic volumetric images in real-time. They can also provide functional information, such as hemoglobin oxygen

concentration and contrast enhancement from exogenous agents. However, the VevoLAZR system uses transducers of high frequency suitable only for small animal research, with low penetration depth. Likewise, typical MSOT systems are targeted more towards preclinical applications. The Nexus 128 is also only for small animal research, and does not support clinical applications. There are many other systems that are being developed. But, they have limitations like, limited use on certain organs like the Twente mammoscope, etc. The Sonix Touch Q+ and Z One PRO have potential for dual modal US-PA clinical imaging owing to their programmable platforms and clinically oriented designs. However, a readily accessible interface for PAI is not presently available for these systems.

Contrast agents

PAI is dependent on the optical absorption of the tissue chromophores. Usually the PA signal is directly proportional to the optical absorption. PAT uses intrinsic contrast from the body including blood, melanin, glucose etc. Fig. 2 shows the optical absorption spectrum of different intrinsic contrast chromophores. In some cases the signal from the intrinsic contrast is not strong enough, therefore, other exogenous or extrinsic contrast agents can be used to enhance the image quality. Moreover, contrast agents can also be used for targeted molecular imaging [50]. Organic dyes, inorganic dyes, nano scale contrast agents, such as gold nanoparticles, nanospheres, nanocages, carbon nanotubes etc. have been explored as PA contrast agents. Among organic dyes methylene blue (MB) and indocyanine green (ICG) have been explored widely as contrast agents [39, 51]. MB is food and drug administration (FDA) approved for sentinel lymph node imaging and has been used for clinical studies. ICG has been used for imaging the urinary bladder, structural imaging of the brain vasculature, sentinel lymph nodes etc. [52]. Different types of nanoparticles including gold nanoparticles,

gold nanorods, carbon nanotubes, nanospheres, liposomes, etc. have been tested as PA contrast agents at different wavelengths [53-57]. Nanoparticle based contrast agents are used for tumor imaging [58]. But they have also been explored for imaging other areas like sentinel lymph node (SLN), brain vasculature, GIT, etc. [59, 60]. The major advantage of using nanoparticles over organic or inorganic materials is that the nanoparticles can be made target specific by coating the nanoparticle surface with specific antibodies [61]. This greatly helps in targeted imaging and therapy especially for diseases like cancer where treating the cancer by not damaging the surrounding tissues is very important. Although so many contrast agents have been tested for PA imaging, only very are FDA approved. Some of the limitations the contrast agents needs to overcome for clinical use are biocompatibility, toxicity, sufficient systemic circulation times, efficient renal clearance, ability for surface modifications with excellent optical absorption.

Figure 2: Optical absorption spectrum of the most commonly occurring chromophores in the body [62].

1.3 Challenges associated with clinical translation

There are many challenges associated with translation of PAT to the clinics. First, the non-availability of commercial ultrasound systems which allows PA imaging. Initially PA imaging was performed with single element transducers, which cannot be used in clinics. Later, modified ultrasound systems which allows PA imaging was developed by companies mostly as research systems. But the problem with such systems was that it does not provide access to raw channel data required for photoacoustic imaging [63]. Additionally, the research systems cannot be used in clinics as it requires FDA approval. Some of these systems does not allow custom programming by the user or are suitable only for small animal research which limits the clinical translation. Only very recently, commercial clinical US imaging systems are available with raw channel data access. Second, the size of the lasers being used. The lasers are expensive, bulky, non-portable, and requires an optical table to house them. Even very tiny movements will lead to misalignment of the laser [64]. Effective light delivery strategy is much needed to develop handheld systems [63, 65]. Third, the pulse repetition rate of the lasers are very low (in the order of few Hz), which affects the imaging framerate [66]. High framerate imaging is required for dynamic photoacoustic imaging, which is not feasible with the current systems. Fourth, the availability of very few FDA approved contrast agents. Although, different contrast agents are being tested for PA imaging, only very few have been FDA approved. There is a huge requirement for many more FDA approved contrast agents for PA imaging. Finally, better reconstruction and image processing algorithms are needed for obtaining better quality PA imaging, which will enable the physicians to get a better understanding.

1.4 Structure of thesis

In **Chapter 2**, we discuss the development of a high framerate (7000 frames per second) clinical PA imaging system. For this we use a pulsed laser diode with pulse repetition rate of 7000 HZ as the light source, and combine it with a programmable, clinical ultrasound imaging system. The high frame photoacoustic imaging (B-mode) measured the flow rate of a fast moving object. So far, the reported 2-D photoacoustic B-scan imaging is only a few tens of frames per second using a clinical ultrasound system. We have demonstrated phantom imaging to view and measure the flow rate of ink solution inside a tube.

In **Chapter 3**, we report an integrated PA and US imaging system by combining the light delivery to the tissue with the US probe. Monte Carlo simulations were used to optimize the probe holder design. Several probe holders were designed for different light launching angles using 3D printer. The simulated results were validated using phantom imaging experiments. The results also validate that Monte Carlo simulation as a tool can be used to optimize the probe holder design depending on the imaging needs.

In **chapter 4**, we discuss about two different applications of our developed handheld dual modal US-PA imaging system. First, we report imaging for SLN and needle guidance in small animal. Methylene blue and indocyanine green were used for mapping the SLN at 675 and 690 nm wavelength illuminations, respectively. Additionally, needle guidance with combined ultrasound and PAI was demonstrated using this imaging system. Up to 1.5 cm imaging depth was observed at a frame rate of 5 fps, which is sufficient for future translation into human SLN imaging and needle guidance for fine needle aspiration biopsy. Second, urinary bladder imaging is shown

in small animals. Structural and functional bladder imaging is shown here with combined US-PA imaging in rats. Structural imaging of the rat bladder is shown using black ink as the contrast agent. Also, the feasibility of photoacoustic imaging for a common bladder disorder, vesicoureteral reflux is studied using urinary tract mimicking phantoms. In addition, non-invasive clearance study of gold nanorods from circulation by monitoring the accumulation of the gold nanorods in the bladder was shown.

In **chapter 5** we explored two different contrast agents for PA imaging. Usage of dual modal US-PA contrast agents are needed for doing dual modal imaging using the same contrast agent. Here, a flow-focusing junction based microfluidic device was used for the generation of nitrogen microbubbles ($<7\text{ }\mu\text{m}$ in diameter) in two photoacoustic contrast agents: methylene blue and black ink (BI). The microbubbles in both solutions were injected into tubes for phantom imaging. Finally, the microbubbles were injected into the urinary bladder of rats for *in vivo* animal imaging. The effect of size and concentration of microbubbles in both MB and BI solutions, on the US and PA signals, has been examined. Finally, near-infrared light-sensitive liposomes coated with gold nanostars (AuNSs) was studied for PA imaging and drug release applications. Upon excitation by laser light at specific wavelength, AuNSs facilitate rapid release of the contents from the liposomes due to local heating and pressure wave formation. We described the design and optimization of the AuNSs-coated liposomes and demonstrate the release of both hydrophobic and hydrophilic model drugs through laser excitation at near-infrared wavelength. The use of AuNSs-coated liposomes as a photoacoustic contrast agent is explored with tissue phantom experiments.

In **chapter 6**, we summarize the work and propose future directions.

Chapter 2

High frame rate photoacoustic imaging¹

2.1 Introduction

Light penetration into biological tissue is greater in the near-infrared (NIR) wavelength region than visible region. Therefore, pulsed lasers emitting NIR wavelength lights have been used for deep-tissue PAT imaging. For the light excitation source, usually, a Nd:YAG pump laser either pumped a dye laser or an Optical parametric oscillator (OPO) laser to generate light in the NIR wavelengths. But, these types of lasers are bulky (often requiring an optical table to house the laser), expensive, and slow (typical pulse repetition rate for such lasers is 10-20 Hz with ~100 mJ of energy per pulse), making the PAT system difficult to translate into clinical setup. Therefore, there is a need to develop PAT system which is compact, affordable, portable and have a higher frame rates [65].

High frame rate PA imaging is also required in areas where a small region, undergoing rapid changes, needs to be imaged fast. The potential applications for such fast PAT system could be imaging of the motion of the heart valves, shape of heart, myocardial functions, deep vein thrombosis or even monitoring circulating tumor cells in blood vessels. Another area where real-time PA imaging can be useful is in the imaging of circulating tumor cells in blood stream (an indicator of the metastasis of cancer). Imaging CTC is most commonly performed *ex vivo* only. However, recently *in vivo* photoacoustic flow cytometry has been demonstrated with the injection of external

¹ Part of the work presented in this chapter has been published in *Biomedical Optics Express*. **K. Sivasubramanian**, and M. Pramanik, "High frame rate photoacoustic imaging at 7000 frames per second using clinical ultrasound system," *Biomedical Optics Express* 7(2), 312-323 (2016).

contrast agent (gold nanoparticles) with good PA contrast [40, 67]. Since, melanoma has strong light absorption in the NIR wavelengths; it does not require any external contrast for the detection. Label free PA imaging of CTC melanoma clusters was demonstrated with a linear array transducer recently where cluster of up to 7 melanoma cells was imaged successfully [68]. Another potential area of application is to image the heartbeat of the rodents; they have approximately 400 beats per minute which can be imaged using the high speed PAT system [69]. Photoacoustic Microscopy imaging was performed at 50 fps to image the microvasculature and heartbeat of the nude mice [70]. However, PAM can only be used for shallow imaging depth. For deep tissue imaging, PAT is preferable.

So far, PAT with only a few tens of frames per second (fps) have been reported in literature. There are two types of PAT system. The first type is based on single element ultrasound transducers, where the transducer rotates around the sample and collects PA signal in a circular geometry. These types of PAT systems are very slow and the mechanical scanning limits its use for clinical applications. Recently, it was shown that low cost, portable PAT systems can be built with reasonable imaging depth (up to 2 cm) with single element transducer [71]. This is the fastest reported PAT system till now with single element transducer with cross-sectional imaging speed of 3 sec. Most traditional PAT system with single element transducer will take several minutes to take one cross-sectional PAT images. Therefore, making high frame rate PAT imaging with single element transducer is very challenging. The second type of PAT system consists of a ultrasound array transducer and a parallel data acquisition system. PAT of the cortical hemodynamics of small animal *in vivo* with a temporal resolution of 1.6 s using a custom build circular array transducer and parallel data acquisition system has been demonstrated [72]. However, such PAT system has limited application due to the

inflexibility of the ultrasound array transducer (circular and fixed size, hence, can be applied only to small animal brain imaging). A far better approach will be to modify a clinical ultrasound imaging to incorporate photoacoustic imaging. As a clinical ultrasound imaging system give the user flexibility of the type of array transducers one can use depending on the application areas. With such systems real time phantom imaging up to 15 mm depth was done at 20 fps. In another study done with the OPO-Ultrasound system, *in vivo* real time imaging of blood vessels in rat was performed at 20 fps [73]. Therefore, the maximum frame rate we found so far from PAT is 20 fps. Apart from the OPO, high frequency pulsed laser diodes (PLD), and light emitting diodes (LED) are also being explored as light sources for PA imaging [74]. Some of the disadvantages of the PLD/LED based PAT system includes limited wavelength tunability, low pulse energy, lower penetration depth of light in tissues. These disadvantages limit the clinical usage of the laser for different applications.

We report up to 7000 fps PAT imaging (B-scans) by use of a low cost, light weight PLD. The PLD has a maximum pulse repetition frequency of 7000 Hz with maximum pulse energy of ~1.4 mJ. The PLD together with clinical ultrasound imaging system enabled very high frame rate PA B-scan imaging. Phantom experiments were conducted to demonstrate the high frame rate imaging capability. The flow of black ink was imaged at high frame rate PA imaging. Various flow rates were used from 3 cm/s to 14 cm/s. From the PA images we were able to measure the flow rate accurately as well. For comparison, a low pulse repetition rate Nd:YAG pumped OPO laser (standard laser used for most PAT system) based PA imaging, and normal ultrasound imaging were also done.

2.2 Methods

2.2.1 PLD-PAT imaging system

The experimental set up is shown in Fig. 3 (a). We have used a clinical research ultrasound system (ECUBE 12R, Alpinion, South Korea) capable of dual modal imaging (both PA imaging as well as ultrasound imaging). To use the system in the PA mode, we need to provide the laser excitation and a controlling unit which can synchronize the laser excitation and ultrasound detection. For the high frame rate PA imaging we used the pulsed laser diode (Quantel DQ-Q1910-SA-TEC), which can provide ~136 ns pulses at a near-infrared (NIR) wavelength of 803 nm, and maximum pulse energy of ~1.4 mJ. It has a pulse repetition rate of 7000 Hz (max). The PLD generates a rectangular laser beam which diverges with an angle of ~11.5, and ~0.65 degree along slow and fast axes, respectively. The PLD is controlled by the laser driver unit (LDU) which consists of a temperature controller (LaridTech, MTTC1410), a 12 V power supply (Votcraft, PPS-11810), a variable power supply (to vary the laser output power), and a function generator (to control the laser repetition rate). The pulse energy and repetition rate can be controlled independently with the variable power supply (BASETech, BT-153), and the function generator (FG250D, Funktionsgenerator), respectively. The function generator provides a TTL (Transistor-Transistor Logic) signal to synchronize the laser excitation and the ultrasound data acquisition by the clinical ultrasound system. A cylindrical lens (CL) was used in front of the laser window to make the laser beam narrower. On the sample the laser beam spot was around 15 mm x 5 mm. The PA signals generated were acquired by a linear array transducer (L3-12 transducer, compatible with the Ecube ultrasound system) consisting of 128 array elements. The probe has a center frequency of 8.5 MHz (95% fractional bandwidth), array element pitch 0.3 mm and elevation height 4.5 mm. The

Ecube system has a 40 MHz sampling frequency and 12 bit Analog-to-digital convertor (ADC) to acquire the data and a data transfer speed of 6 Gb/s. Fig. 3 (b) shows the picture of the experimental set up with all the components. This setup is called as PLD-PAT system.

Figure 3: (a) Schematic of the PLD-PAT ultrasound setup; PLD: Pulsed laser diode, UST: ultrasound array transducer, CL: cylindrical lens, SP: syringe pump, LDU: laser driver unit, USM: clinical ultrasound machine. (b) Image showing all the components of the PLD-PAT imaging setup for flow imaging. (c) The L3-12 linear array ultrasound transducer which can be operated in 2 different modes. One using all the 128 arrays elements, and the other using 64 channels elements only (element numbers 1-64 or 65-128).

2.2.2 OPO-PAT imaging system

A similar set up was done with OPO laser as light excitation source, which is typically used for most PAT systems for deep tissue imaging. Since these types of lasers have

lower repetition rates (typically 10-100 Hz), but higher pulse energy, they cannot provide very high frame rate PAT images (the maximum frame rate one can obtain is same as the laser pulse repetition rate *i.e.*, 10-100 Hz). The excitation laser consists the OPO (Continuum, Surelite OPO) laser pumped by a 532 nm Nd:YAG pump laser (Continuum, Surelite Ex). The OPO generates 5 ns duration pulses at 10 Hz repetition rate (maximum) with wavelength tunable from 680 nm to 2500 nm. For the experiments it was tuned to 803 nm since the PLD also operates at that wavelength. The laser beam spot size falling on the sample was ~1.5 cm in diameter. Like before the PA images were captured by the L3-12 linear array transducer in the Ecube ultrasound imaging system. The trigger out from the pump laser synchronized the data acquisition on the Ecube system. This setup is called as OPO-PAT system.

2.2.3 PA data acquisition and image formation

To operate the Ecube ultrasound imaging system in the PA mode, one needs to use the research mode. In the research mode, the instructions can be written using a python script to set the parameters like imaging depth, data acquisition modes, number of frames to be acquired, type of data to be saved etc. The PA signal generated by the laser excitation is acquired by the array transducer L3-12 (128 element linear array with center frequency of 8.5 MHz, 95% fractional bandwidth, array element pitch 0.3 mm, and elevation height 4.5 mm). However, the ultrasound system has 64 parallel data acquisition hardware. Fig. 3(c) shows how the data acquisition in photoacoustic mode works. The data acquisition is done by 64 channels of the receiver each time. This is fixed and cannot be modified. Therefore, in one single laser pulse the PA signals from 64 array elements can be captured. To collect all the 128 array element data we need 2 laser pulses. Thus the system can be operated in two different modes. When operated in mode 1, there are two frames per PA image (*i.e.*, the 64 channel receiver operates

twice which requires two trigger from the laser), thus the effective frame rate is half the laser repetition rate. For example, if the PLD was set to run at 7000 Hz, the effective frame rate for PA B-mode imaging was 3500 fps ($7000/2 = 3500$). When OPO was used as excitation source we could only achieve 5 fps (OPO runs at 10 Hz repetition rate) PA imaging in this mode. On the other hand in mode 2, only half of the transducer array elements (64 elements) can be used and it requires only a single laser trigger to capture a B-mode PA image. Thus, the frame rate of the PA images is same as the laser pulse repetition rate. In this mode, we could achieve a maximum frame rate of 7000 fps using PLD and 10 fps using the OPO laser. The acquired PA signals then go through a series of inbuilt filters for the image processing. It also allows the user to add and custom modify the filter module to suit for the individual needs. No custom made filters were added for this work. The signal first enters the source filter and gets processed. It moves to the custom filter adapter, and then to the B-filter. B-filter is followed by the scan conversion filter, and finally the render filter. The output from the render filter is displayed on the Ecube monitor as PAT images. At the same time these beamformed and scan converted PA data can also be saved on the hard drive of the Ecube system. The raw channel data can be saved into the hard drive as well. For this work only beam formed and scan converted PA data is saved. We have chosen to save a total of 8000 frames (B-scans) when PLD was used as excitation source, and 20 frames when OPO was used as excitation source.

2.2.4 Ultrasound imaging

For comparison ultrasound imaging was also done. In the ultrasound mode, the ultrasound machine captures the B-mode ultrasound data and after beamforming and scan conversion the images are displayed on the screen of the Ecube system. For the ultrasound imaging there is no need for external triggering, as the system operates on

internal triggering. Thus the external trigger was removed. The L3-12 ultrasound transducer performed both transmitting and receiving of ultrasound waves. All the ultrasound data (RF signals, IQ data, Beam formed data, and Scan converted data) can be saved in the hard drive of the Ecube for post processing if needed. However, no post processing was done for the ultrasound images.

2.2.5 Phantom experiments

To demonstrate the capability of the imaging system providing high frame rate PA imaging, we carried out flow imaging. A low density polyethylene (LDPE) transparent tube of inner diameter 1.15 mm was placed in the water bath. The tube was positioned such a way that the laser beam falls on it. Black ink (which has good optical absorption at 803 nm wavelength and can produce PA signal) was injected into the tube using a 10 mL syringe with a 24G needle. The optical absorption of the black ink is $\sim 5 \text{ cm}^{-1}$, very similar to that of blood, but less than Indocyanine Green at 803 nm excitation wavelength [75]. The flow rate of black ink was controlled with a programmable syringe pump (AL-1000, World precision instruments). The transducer was placed at a distance of 1 cm from the tube with a total imaging depth of 2 to 3 cm. The ink flow rates inside the LDPE tube used for the experiments were 3 cm/s, 6 cm/s, 10 cm/s, and 14 cm/s. The specified flow rates were chosen to demonstrate that with the high frame rate PA imaging system we can image and accurately find the various flow rates from the PA images.

The flow rate of the ink flow was calculated from the saved PA images. The distance travelled by the ink is measured from the high contrast of the ink in the PA B-scan images. The PA image (beamformed) has a pixel size of 0.03 cm. The pixel size was determined by the beam former parameter. From the saved PA image the distance

moved by the ink in a given period of time was calculated. A start frame and an end frame was chosen, and if the ink flow moved by a distance d in N frames ($N = \text{end frame number} - \text{start frame number}$), then the flow rate is calculated as, $\text{Flow rate } (F) = d/N$.

2.2.6 Laser Safety

When PAT is used to image subjects *in vivo*, the maximum permissible pulse energy and the maximum permissible pulse repetition rate are governed by the ANSI laser safety standards [76]. The safety limits for the skin depend on the optical wavelength, pulse duration, exposure duration, and exposure aperture. In the spectral region of 700-1050 nm, the maximum permissible exposure (MPE) on the skin surface by any single laser pulse should not exceed $20 \times 10^{2(\lambda-700)/1000} \text{ mJ/cm}^2$ (λ is the wavelength in nm). Therefore, at 803 nm the MPE for a single pulse exposure is $\sim 31 \text{ mJ/cm}^2$. However, for our imaging purpose the exposure time was roughly 2 seconds with multiple laser pulses. The MPE for exposure time $t = 2 \text{ sec}$ is given by $1.1 \times 10^{2(\lambda-700)/1000} \times t^{0.25} \text{ J/cm}^2 (= 1.1 \times 10^{2(803-700)/1000} \times 2^{0.25} \text{ J/cm}^2 = 2.07 \text{ J/cm}^2)$ [43]. Since the PLD was operating at 7000 Hz (total number of laser pulses in 2 sec = $2 \times 7000 = 14000$), the MPE becomes 0.15 mJ/cm^2 ($2.07/14000$) per pulse. In our imaging system, the pulsed diode laser provided maximum $\sim 1.4 \text{ mJ}$ pulse energy, and the laser beam was spread over an area of $\sim 0.75 \text{ cm}^2$ ($\sim 1.5 \text{ cm} \times 0.5 \text{ cm}$). Therefore, the laser fluence on the sample surface was $\sim 1.9 \text{ mJ/cm}^2$ ($1.4/0.75$). Thus, in the PLD-PAT imaging system the laser fluence on the sample surface was above the MPE.

For OPO excitation, the OPO laser energy output was at $\sim 40 \text{ mJ}$ per pulse at 803 nm on the sample surface ($\sim 1.5 \text{ cm}$ diameter spot). Thus the fluence on the sample surface was $\sim 22.6 \text{ mJ/cm}^2$. Thus the fluence is within the ANSI safety limit.

The MPE safety limit was not strictly followed in case of phantom imaging with the PLD-PAT system in this work. Moreover, the aim of this study was to demonstrate the possibility of high frame rate imaging at 7000 fps. Thus, no frame averaging was not done to improve the signal-to-noise (when the laser is operated at lower energy the PA signals will be weaker and averaging will be required). For *in vivo* experiments, the fluence on the sample surface can be reduced by various ways – a) spreading the beam over a larger area, b) reducing the pulse repetition rate, c) reducing the exposure time less than 2 sec, or d) reducing the laser power output by controlling the power supply itself. Among the four, reducing the pulse repetition rate or reducing the exposure time is the best way to optimize the SNR and still be within the ANSI safety limit. The SNR decreases linearly with the energy of the pulses, whereas averaging more pulses only increases the SNR by square root of the number of averages. Therefore, it is preferable to use lower frame rates to maximize SNR, which is particularly important for the clinical translation [77]. On the other hand, reducing the exposure time will also help to be within the ANSI safety limit. However, if one needs to do continuous monitoring using PA imaging for long time, this may not be feasible.

2.3 Results and Discussion

As described earlier, a tube was used with black ink flowing through it with a specified flow rate. At first it is shown that with just ultrasound imaging it is not possible to see the ink flowing, as ink does not have any ultrasound contrast. However, ink is a good optical absorber and will be visible in photoacoustic imaging. Fig. 4 shows the screenshot of the ultrasound B-mode image obtained with the Ecube. A depth of 2 cm was imaged and the transducer was placed at a distance of 1 cm from the tube with ink flow. The flow rate of the black ink was maintained at 3 cm/s. The tube part of the image alone is enlarged and shown on the right. Only the LDPE tube boundaries are

visible in the ultrasound image. Obviously the flow of the ink inside the tube is not visible as ink does not have good ultrasound contrast.

Figure 4: Ultrasound B-mode image on the ECUBE system showing the flow of the black ink inside the tube. The enlarged tube region shows that we can see the boundary of the tube, but no flow can be visible in the ultrasound images, as ink does not have a good ultrasound contrast.

PA imaging was done for the different flowrates at the two different modes of the data acquisition as discussed previously. Figs. 5 and 6 show the PA B-scan images obtained from the PLD-PAT system. Fig. 5 shows the flow of the ink in the tube at various given time points when the transducer was operated in mode one. In this mode 3500 fps PA images were recorded. Fig. 6 shows the same PA images when the transducer is operated in mode two. In this mode 7000 fps PA images were recorded. The PA images represented here are at time points $t = 0$ s [Figs. 5(a), 5(e), 6(a), 6(e)], 0.05 s [Figs. 5(b), 5(f), 6(b), 6(f)], 0.1 s [Figs. 5(c), 5(g), 6(c), 6(g)], and 0.25 s [Figs. 5(d), 5(h), 6(d), 6(h)]. For representative purposes only two different velocities are shown here 3 cm/s [Figs. 5(a-d), 6(a-d)], and 14 cm/s [Figs. 5(e-h), 6(e-h)]. It is clearly seen that the ink is flowing through the tube from both Figs. 5 and 6. 175 frames are being recorded in 0.05 s when operated at 3500 fps and 350 frames are being recorded in 0.05 s when operated at 7000

fps. The set of images here show the ink flowing inside the tube for a time period of 0.25 s. From the images, the gradual flow of the ink can be observed at different time points. The distance moved by the ink is approximately 15 mm which is consistent with the laser beam width. However, depending on the ink flow rates the time taken to move that distance are different, which can be observed from the images.

Figure 5: Set of PA images showing the ink flow at various time points obtained with PLD-PAT system. PA imaging was done at a frame rate of 3500 fps. Schematic of the setup is shown on the top. (a-d) Flow rate was 3 cm/s; (e-h) flow rate was 14 cm/s. All images have same scale bars shown in (a). The normalised colourmap is shown on the right.

Figure 6: Set of PA images showing the ink flow at various time points with the PLD-PAT system. PA imaging was done at a frame rate of 7000 fps. (a-d) Flow rate was 3 cm/s, (e-h) flow rate was 14 cm/s. All images have same scale bar shown in (a). The normalized colourmap is shown on the right.

The same sets of experiments were carried out with OPO-PAT system. As mentioned before, when the Ecube was operated in mode one, PA images was recorded at 5 fps and when the Ecube was operated in mode two, PA images were recorded at 10 fps. The PA images represented here are at time points $t = 0$ s [Figs. 7(a), 7(c), 7(e), 7(g)], 0.02 s [Figs. 7(b), 7(d)], 0.1 s [Figs. 7(f), 7(h)]. For representative purposes only two different flow rates are shown here 3 cm/s [Figs. 7(a,b,e,f)], and 14 cm/s [Figs. 7(c,d,g,h)]. The OPO-PAT system at best can take PA images at 0.1 s interval (10 fps). Thus the two time points at which PA images were captured at $t = 0$ s and $t = 0.1$ s (for 5 fps PA acquisition the frames are at $t = 0$ s and $t = 0.2$ s). It can be observed that the ink has flowed distance of approximately ~ 15 mm which is the total imaging window for the OPO laser beam within just 1 frame and the flow of the ink can't be visualized as was observed with PLD-PAT system (as evident in the PA static images).

Figure 7: Set of PA images showing the ink flow at various time points as obtained by OPO-PAT system. (a-d) PA imaging was done at a frame rate of 5 fps and (e-h) at a frame rate of 10 fps. (a,b,e,f) Flow rate was 3 cm/s, (c,d,g,h) flow rate was 14 cm/s. All the images have same scale bars as shown in (a). The normalized colourmap is shown on the right.

To substantiate that from the high frame rate PA images we are getting the flow measurement correctly, we processed the saved PA data into MATLAB. Table 1 shows the comparison between the actual flow rates (as controlled by the syringe pump) with the flow rates calculated from the PA images. For example, when the ink flow rate is 3

cm/s (as recorded from the syringe pump) at 3500 fps PA imaging, the ink moves a distance of 2.1 mm in 0.057 seconds (*i.e.*, 200 frames recorded), thus the flow rate as calculated from the PA image is 3.7 cm/s. Similarly, calculations were done for the all the other flow rates PA data. From the comparison in table 1 it can be observed that the actual flow rate and the calculated flow rate from the PA images are very consistent for both 3500 fps and 7000 fps PA imaging.

Table 2: Comparison of the flow rates as controlled from the syringe pump and measured from the PA images from the PLD-PAT system

Frame rate	Measured flowrate (cm/s)	Calculated flowrate (cm/s)
3500	3	3.7
3500	6	6.5
3500	10	10.5
3500	14	14.7
7000	3	3.6
7000	6	6.3
7000	10	10.5
7000	14	14.7

The maximum possible frame rate with the current PLD-PAT system is 7000 fps. This is mainly constrained by the excitation laser source pulse repetition rate, data acquisition modes, imaging depth etc. Up to 14 cm/s ink flow rate PA imaging was demonstrated in this work. However, a much higher flowrates can be visualized with the system. For the data acquisition mode 2, only 64 channels were used for PA signal acquisition. The distance of each pixel on the PA image is 0.3 mm. From one frame to the next frame, one can measure a maximum distance of up to $64 \times 0.3 \text{ mm} = 19.2 \text{ mm}$.

Hence, the theoretical maximum flowrate that can be captured by this system is 134.4 m/s [19.2 mm x 7000 /s]. When the imaging is done in mode 1, two frames needs to be acquired to obtain a single PA image, one can image a larger area with this mode, whereas with the mode 2 since only one frame is captured for a single PA B-mode image the imaging area will be half of that in mode 1. But, for applications which require imaging to be done in a small area at a high framerate mode 2 is more preferred. The frame rate can be increased further if lasers are available with higher repetition rate. At present, the maximum imaging depth is $(1/7000)*1540 \text{ m/s} = \sim 22 \text{ cm}$. This depth is far more than what photoacoustic imaging can reach at the moment. The current system has certain drawback as well. The linear array transducer has a limited field of view. As a result it introduces artefacts in comparison to the circular array/spherical array transducer.

2.4 Conclusion

It was demonstrated that high frame rate PA imaging is possible using the PLD-PAT clinical ultrasound system. A frame rate of 7000 fps PA B-scan imaging is feasible. With such a high frame rate of 7000 fps flow rate up to 134.4 m/s (theoretical) can be captured. It was shown that the flow can be visualized as well as accurate flow measurements can be done from the PA B-mode images obtained from the PLD-PAT system with various flow rates 3 cm/s to 14 cm/s. The PLD-PAT clinical system has a few limitations and challenges, such as limited view artefacts (caused by the linear array transducer), multiple acoustic reflections degrading the image quality, poor signal-to-noise due to lower laser pulse energy, and integrating the laser light source inside the ultrasound probe. However, the PLD being low cost, portable, and easy to handle may be easier to translate to clinical applications.

Chapter 3

Handheld probe for dual modal US-PA imaging²

3.1 Introduction:

High framerate imaging is beneficial for dynamic PA imaging. But, the energy from the pulsed laser diode is not sufficient for deep tissue imaging. Therefore, high energy lasers are needed for deep tissue imaging. Conventionally, for PA signal detection, single element ultrasound transducer based PAT scanners requires mechanical scanning and hence slow. Such, PAT systems will be less preferred in a clinical setup [71]. Therefore, clinical ultrasound array transducers are better suited for clinical applications. Clinical ultrasound imaging systems consists of different types of array transducers (linear, convex, phased array) and allows the user to choose based on the area/region of imaging. Therefore, integrating PA imaging with the clinical ultrasound system is a better design for clinical translation of PA imaging. Additionally such a system also provides dual modality ultrasound as well as PA imaging capability, which will be very useful for clinicians. Multimodal PA ultrasound fluorescence imaging (PAUSFI) system combining PA, ultrasound, and fluorescence imaging was reported earlier [78]. However, such system may find limitations in acceptance to the clinical community, since it is not integrated with the clinical ultrasound imaging platform. To use clinical ultrasound imaging platform for PA imaging one needs to find ways to integrate the light delivery with the ultrasound probe. One can achieve this by modifying the ultrasound transducer itself internally to incorporate the laser light

² Part of the work presented in this chapter has been published in *Journal of Biomedical Optics*. **K. Sivasubramanian**, V. Periyasamy, K. K. Wen, and M. Pramanik, "Optimizing light delivery through fiber bundle in photoacoustic imaging with clinical ultrasound system: Monte Carlo simulation and experimental validation," *Journal of Biomedical Optics* 22(4), 041008 (2017).

delivery. A pulsed laser diode was integrated inside the linear array transducer to develop an integrated photoacoustic ultrasound imaging system (PA-US) [65]. The energy of the light source is still quite low for deep tissue imaging (only small diode laser sources can be housed inside an ultrasound probe). Moreover, this involves modification of the ultrasound transducer internally during the probe manufacturing process, leading to a custom made probe. Hence, the cost associated with it is high. The other, more practical approach is to externally integrate the laser light delivery with the ultrasound probe. This method was used for the guided needle biopsy of the sentinel lymph nodes for both pre-clinical and clinical imaging [63, 73]. With this system it was shown that PA imaging can be done up to a depth of 5.2 cm [79]. A clinical ultrasound machine was used for combined PA-US imaging at different depths for different types of transducers (linear array, convex array, phased array, and endocavity). Light was coupled externally to the transducer from a portable OPO laser with a customized adapter. *In vivo* PA imaging was done in small animals and human forearm using a contrast agent [37].

The design of this probe holder, which combines the light delivery and the ultrasound probe, is critical for optimizing the light delivery to the target area. There are several factors affecting the light reaching the target object inside the tissue, such as, the angle of light delivery, the distance between the ultrasound transducer and the fiber, the distance between the fiber tip and the tissue surface, optical properties of the tissue and the target object etc. Using a clinical PA imaging set up, the irradiation distance was optimized for 3D scanning of the human forearm [80]. Light was coupled externally with a bifurcated fiber bundle at varying angles (20° to 80°) for PA imaging on tissue mimicking samples. The fiber was placed on one side of the transducer using a clip. The best angles were found to be 40° to 50° for imaging depth of 1 to 2 cm [81]. The

angle of illumination depends on the distances at which the fiber and the ultrasound probe are placed [82]. In this work we optimize the angle of light delivery from both sides of the ultrasound probe using Monte Carlo simulation for light propagation in tissues with embedded sphere (MCES). Experiments were carried out for different imaging depths using a clinical PA imaging setup. In the simulations apart from the light launching angles, the fiber to tissue distance and the fiber to ultrasound probe distance were also considered. No work has been reported till now on the optimization of the probe holder design using Monte Carlo simulation as a precursor for light delivery from both the sides of the ultrasound probe.

The probe holder design may also vary depending on the area/organ being imaged. Here we focus on sentinel lymph node imaging. PA imaging for noninvasive SLN identification is a promising tool for breast cancer staging [39]. Sentinel lymph node biopsy (SLNB) is commonly used for staging of breast cancer. To visualize the lymph nodes radioactive colloids together with methylene blue dye are commonly used. Noninvasive SLN imaging along with percutaneous fine needle aspiration biopsy will reduce the morbidity associated with SLNB. We used MCES to study the light delivery angle for a range of imaging depths of SLN [83-85]. Monte Carlo code by Wang et al [86] was modified for fiber source illumination and to handle refractive index mismatch of embedded sphere (SLN is modelled as a sphere in this work) and tissue. Previously, Monte Carlo simulations were reported for light optimization for breast imaging, however, inclusion of different types of embedded objects (sphere in this case) was not considered. Initially (study 1), the effect of fiber-to-probe distance (FPD) was computed for different angles of illumination (0° to 89°) for a fixed SLN depth. Then (study 2), we considered the light hitting the transducer (the photons were terminated to keep it simple), which mimics the experimental set-up better. Next (study 3) focused on the

effect of fiber-to-tissue distance (FTD) for various light launch angles. Lastly (study 4), for a fixed FPD and FTD, the SLN depth inside the tissue was varied and simulations were done for various light launching angles. Reflection of diffuse reflected light back into the tissue by fiber bundle face and probe surface (in contact with tissue) was also considered in studies 2-4. Four different probe holders were designed, which were printed using a 3D printer. Later, experiments were carried out mimicking the simulation setup (study 4).

Table 3: Optical properties of various elements used in the MCES at 675 nm wavelength.

Layer	Refractive index – n	Absorption coefficient - μ_a (cm ⁻¹)	Scattering coefficient - μ_s (cm ⁻¹)	G	Thickness (cm)
Water	1.33	0.00439	0.000007	1.0	1.0
Chicken breast tissue	1.4	0.1010	25	0.9	2.0 to 4.5
Methylene blue	1.3	1.7049	25	0.9	1.0 (Diameter)

3.2 Methods

3.2.1 Monte Carlo simulation for designing the probe

Monte Carlo simulation for light propagation in multi-layered tissue (MCML) written in ANSI C was modified to embed the SLN inside the tissue layer [86]. The modifications in the code are explained in more detail in the appendix. Figure 1(a) illustrates the cross sectional view of the simulation geometry at the Y = 0 plane, across XZ axis. Table 1 lists the optical properties used for the simulations [87-90]. The ultrasound probe (4 cm x 1 cm along XY axis) extends into the first layer (water), to contact the tissue. Modifications were also made to simulate the light delivery using the bifurcated fiber bundle, where each end has 800 fiber tips distributed over a plane of 4

cm x 0.142 cm. The fibers of diameter 200 μm (with cladding, core diameter is 185 μm) were randomly packed during fabrication. Hence, for simulation the fibers were randomly placed in the rectangle of 40 mm x 1.42 mm with a minimum distance of 200 μm between the fibers. 2 sets of 800 fiber tips were distributed for two fiber bundles. From the generated fiber tips, the minimum distance between the fiber tips was calculated as 200 μm , the maximum separation distance between the fibers was 338 μm , the mean (std) of minimum distance was 228 (25) μm . The angular distribution of photons exiting the fiber was modeled as a function of its numerical aperture (NA). Further explanations are given in the appendix on how the photons were launched from the fiber bundle. For angular launch from the fiber bundle, the direction cosines of the photons were varied according to the launching angle. Figs. 8(b-f) demonstrate the angular launch of the photons for 0° to 60° launching angles, where FPD is 2 cm, FTD is 1 cm. At higher launching angles one can see that the photons start hitting the transducer placed in between the fibers [Fig. 8(f)]

Fiber tips were at $Z = 0$ plane and the FPD was varied from 1 cm, 2 cm, and 3 cm for a SLN (sphere) placed at a depth (thickness of tissue above sphere) of 1.5 cm. In study 1 and 2 the angle of illumination was varied from 0° to 89° in steps of 15° , and FTD was a constant of 1 cm. Study 1 was designed to understand the variation of the absorbance in sphere with respect to angle of illumination and change in FPD, when none of the photons were blocked. On contrary, in our experimental set-up, the ultrasound probe was placed in-between the bifurcated fiber bundles. Hence, in simulation the photons which hit the probe were terminated as seen in study 2. Since the light delivery on tissue is also a function of the FTD, in study 3 FPD was fixed at 2 cm and sphere depth is maintained at 1.5 cm whereas angle of illumination was varied as 0° , 5° , 10° , 15° , 20° , 30° , 45° , and 60° and FTD was 1 cm, 2 cm, 3 cm and 4 cm. All the simulations were

done with a semi-infinite simulation set-up, but in the experimental set-up the size of the tissue medium was finite. In study 4 we considered finite tissue boundaries. For simplicity, the photons which cross the boundary were terminated. In study 4, the angle of illumination were 0° , 5° , 10° , and 15° .

Figure 8: Monte Carlo simulation geometry and fiber based light illumination design. (a) Schematic diagram of the simulation geometry across the $Y = 0$ plane. The optical fiber is at a distance of 1 cm on top of the tissue surface where each end has 800 fiber tips. The sentinel lymph node (sphere with methylene blue here) is embedded inside a semi-infinite tissue medium. Optical properties used in the simulations are given in Table 1. UST: ultrasound transducer, FPD: fiber-to-probe distance, FTD: fiber-to-tissue distance. (b-f) shows the laser beam launched on tissue at angles 0° , 15° , 30° , 45° , and 60° . The distance between the fiber bundles is 4 cm. The views are at azimuthal angle 60° and elevation angle 30° .

Optical properties of the medium such as, absorption coefficient (μ_a), scattering coefficient (μ_s), scattering anisotropy (g) and refractive index (n) determine the propagation of the photon [87, 91]. Here, equal numbers of photons were launched from each fiber tip. Total absorbance in the sphere and distribution of absorbance in 3-dimensional XYZ grids were recorded. Total absorbance in the sphere is independent of the element size chosen in the simulation. For the distribution of absorbance element size (grid size) of $75 \mu\text{m}$ was used. PA signal generated from the SLN is directly

proportional to the absorbance in the sphere. Thus, the absorbance inside the sphere was a good indicator of the strength of the generated PA signal. MATLAB (MathWorks, Massachusetts, USA) was used for generation of the absorption maps.

Studies 1-3 were run for 6.4 million photons (4000 photons per fiber tip). Study 4 was run for 16 million photons (10000 photons from each fiber tip). Convergence analysis was done and the standard deviation of the absorption in the sphere was less than 0.25% when 6.4 million photons and less than 0.15% when 16 million photons were used. Each simulation took ~10 minutes (for 16 million photons) to run on the desktop. The 3D design of fiber holder was such that the distance between the two fiber bundles were not same for different angles of illumination. For 0° holder the fiber to fiber (center to center) distance was 3.8 cm, for 5° and 10° angles the distance was 3.4 cm, and for 15° the distance was 3.6 cm. These distances were used in simulation of study 4 for consistency between simulation and experimental set-up. The thickness of the tissue layer above the sphere was varied from 0.5 cm to 3.0 cm in steps of 0.5 cm (whereas with respect to $Z = 0$ plane the center of the sphere varies from 4.0 cm to 6.5 cm) in study 4.

In experimental cases diffuse reflected photons from tissue are reflected back into the tissue by surfaces such as stainless steel metal in fiber bundle and the ultrasound transducer. Considering the above reflecting surfaces, 36% of the diffused reflected photons are diverted back into the medium in studies 2-4. This reflection of photons in simulation happens at the $Z = 0$ plane. All simulations were done in 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 Photoacoustic and optical spectrum of methylene blue

Since methylene blue is clinically approved contrast agent for sentinel lymph node imaging, here we have used the same for all our experiments. From the literature MB has an absorption peak around 668 nm, however, the OPO laser used here has a minimum wavelength of 670 nm. In order to find an optimum wavelength to work, we first recorded the PA spectrum of the MB in the NIR wavelength range the OPO operates. The MB (Sterop, Belgium) was injected into a transparent low density polyethylene (LDPE) tube of diameter 1.15 mm and placed in water bath. A single element ultrasound transducer (3.5 MHz center frequency) was used to record the PA signal. The signals were first amplified, and then band pass filtered by ultrasound signal receiver (R/A/F) unit (Olympus-NDT, 5072PR), and then digitized and stored in a desktop with data acquisition (DAQ) card (Gage, compuscope 4227). The wavelength of the laser was varied from 670 nm to 870 nm with a 5 nm step size. Each of the obtained PA signals was averaged 10 times. The peak-to-peak PA signal intensity is plotted against the wavelengths. The PA signals were normalized with the laser power. For comparison the optical spectrum of methylene blue with the UV-visible spectrophotometer (Spectramax M5) was done for the same wavelength range. The concentration of methylene blue used is 0.0004 mg/mL.

3.2.3 Design of the fiber bundle holder

The 3D design of the optical bundle holder was done using a computer-aided design (CAD) software package Creo Elements/Pro (Parametric Technology Corporation, Massachusetts, USA). The current designs of the holder is inexpensive and easy to fabricate because they are designed as a single piece without any overhanging features and eliminates the need for assembly of parts. The images of the bundle holder design for the various angles 0°, 5°, 10°, 15° are shown in Figs. 9(a-d). These angles were

chosen keeping in mind the practical difficulties due to the fiber flexibility and length. The holders were fabricated using 1.75 mm diameter polylactic acid (PLA) filament in a desktop 3D printer (MakerBot Replicator 2, MakerBot Industries, Brooklyn, USA). Replicator 2 printer settings for the various parameters were as follows, high resolution, 15% infill with the extruder temperature at 230° C and extruder speed of 90 mm/s. The fiber holder was designed with two slots to hold two ends of the fiber and a single slot to accommodate the ultrasound transducer. The variations in distance between the fiber-bundles for each of the angles were taken into consideration for simulation. The holder was designed for different angles at which the fiber bundle is fixed. The various angles used for the experiments are 0°, 5°, 10°, and 15°. The images of the same are shown in Figs. 9(e-h), respectively.

3.2.4 PAT imaging system

The PAT experimental set up is shown in Fig. 9(i) and an enlarged image of the imaging area is shown in Fig. 9(j). The clinical research ultrasound system (ECUBE 12R, Alpinion, South Korea), capable of dual modal imaging (both PA imaging and ultrasound imaging), was used in this study. The working of the clinical research system is described in section 2.2.1 and 2.2.3. The excitation laser used here was an OPO (Continuum, Surelite OPO) pumped by a frequency doubled nanosecond pulsed Nd:YAG pump laser (Continuum, Surelite Ex). The OPO generates 5 ns duration pulses at 10 Hz repetition rate with wavelengths tunable from 670 nm to 2500 nm. For the imaging experiments the laser was tuned to 675 nm. The laser output from the system was delivered to the sample by coupling through a one meter long optical fiber bundle (Ceramoptec GmbH, Germany) containing 1600 fused multimode fibers. The photograph of the fiber bundle is shown in Fig. 9(k). A lens (L) was used to focus the light to input end of the optical fiber bundle (OF). The fiber bundle was bifurcated

towards the end and light output is delivered through two bundles, rectangular in shape and the area from which light is delivered is 4 cm x 0.142 cm. Each of this area comprises of 800 fiber tips. Each fiber has a core diameter of 185 μm (200 μm with cladding) with a numerical aperture of 0.22. The input and the output ends of the fiber are shown in Fig. 2(l). The two output ends of the fiber bundle are fixed in the custom made probe holder. The energy at the input and the output ends of the fiber was measured and the coupling efficiency of the fiber was ~65%. The light energy at the output side of the fiber bundle was ~32 mJ per pulse at 675 nm. The radiant exposure on the sample surface was estimated to be ~6.4 mJ/cm² (total spot size of illumination from both the fiber bundles is ~5 cm²). Thus the radiant exposure was within the ANSI safety limit of 20 mJ/cm². The ultrasound probe was also fixed inside the fiber holder along with the two fiber ends to make a handheld photoacoustic and ultrasound probe. The lateral and horizontal views of the assembled probe are shown in Figs. 9(m,n). The trigger out from the pump laser synchronizes the data acquisition on the Ecube system. The PA images are displayed on the Ecube screen at a frame rate of 5 fps, since two laser shots are required to obtain one PA image.

3.2.5 Resolution characterization

The axial resolution can be calculated by imaging a point target at different depths. A 23 G needle (0.6 mm) was used as the point target and imaged at depths of 1 cm, 1.5 cm, 2 cm, 2.5 cm, and 3 cm. To determine the axial and lateral resolution, the point-spread function is taken from the normalized PA signals along the respective directions and fitted into a Gaussian distribution function. The full width at half maximum (FWHM) is obtained. There is another approach of obtaining a point spread function when imaging a point target is difficult (as in our case, for a very tiny point

Figure 9: (a-d) The drawings used to fabricate the fiber holder with various angles of light delivery, 0° , 5° , 10° , and 15° , respectively. (e-h) Photographs of the 3D printed fiber holders. (i) Photoacoustic imaging system with clinical ultrasound platform. OPO - optical parametric oscillator, OF - optical fiber bundle, FH - fiber holder, USM - clinical ultrasound machine, L - lens. (j) Zoomed section of the handheld probe and imaging sample, showing the fiber holder with the optical fibers and transducer. UST - ultrasound transducer, MB - spherical phantom with methylene blue embedded in tissue and placed in water bath. (k) Photograph of the fiber bundle. (l) Photograph of the input and the output ends of the fiber bundle. The two rectangular parts constituting the output end of the fiber bundle and the input end is of a circular shape. (m) Top view of the fiber holder with the optical fibers and the transducer attached. (n) Front view of the fiber holder with the optical fibers and the transducer attached. (o) Chicken breast tissue with the embedded spherical phantom filled with methylene blue. (p) Photograph showing the phantom stacked with 2.5 cm of chicken tissue.

Figure 10: Axial resolution characterized at different depths calculated from the full width at half maximum.

target the signal is quite small and therefore we use a slightly larger target). If the target is large, instead of directly getting the point spread function, one can obtain an edge spread function. Then by taking the first derivative of the edge spread function, one can get the point spread function and calculate the resolution from that. Fig. 10 shows the plot for axial resolution at different depths. The PA signal along the axial and lateral direction was plotted and fitted to a Gaussian distribution function. The full width at half maximum was calculated at various depths of 1 cm, 1.5 cm, 2 cm, 2.5 cm, and 3 cm. The plot for axial resolution is shown in Fig. 1(e). The axial resolution was calculated to be $207 \pm 45 \mu\text{m}$. The lateral resolution is limited by the element pitch of the UST. Theoretically, the lateral resolution is $300 \mu\text{m}$, which is the element size of the UST. The lateral resolution calculated from the acquired PA image of the needle was $351 \mu\text{m}$.

3.2.6 Phantom experiments

Imaging with phantoms mimicking SLN was performed. A spherically shaped transparent object was prepared with commercially available latex material similar to gloves of $50 \mu\text{m}$ thickness and filled with 0.5 mL of methylene blue. It was knotted manually to seal in the shape of a sphere of 1 cm diameter to mimic the SLN. The spherical object was completely filled with methylene blue dye at a concentration of 10 mg/mL. The spherical phantom was embedded in a chicken breast tissue, approximately 3 cm in thickness. For imaging at various depths, commercially available chicken breast tissue was cut manually in the size 10 cm x 7 cm and 0.5 cm in thickness. Five such slices were cut and stacked one by one to increase the depth of imaging from 0.5 cm to 2.5 cm in steps of 0.5 cm. The photograph of the spherical phantom embedded in the tissue is shown in Fig. 9(o). The SLN phantom along with the chicken breast tissue was placed in water bath on which the handheld ultrasound-laser probe was placed to

acquire PA images at different depths and at different angles of the light launch (by using different probe holder) at FTD 3 cm. The different angles used for light delivery are 0° , 5° , 10° , and 15° . The different depths for which phantom imaging were performed are 0.5 cm, 1 cm, 1.5 cm, 2 cm, and 2.5 cm. The photograph of the phantom with multiple layers of tissue is shown in Fig. 9(p).

3.3 Results and discussion

Since MB is used clinically for SLN imaging, it has been used as contrast agent for this study. MB has a peak light absorption at 668 nm. The UV-visible spectrum of methylene blue is shown in Fig. 11(a). Since the OPO provides laser output from 670 nm, it was first checked that the MB can produce strong PA signal near or above 670 nm. The MB PA spectrum is shown in Fig. 11(b). Note that the maximum peak-to-peak PA signal intensity is at 670 nm. The PA spectrum is in correlation with the absorption spectrum of methylene blue. Although the highest peak-to-peak PA signal was obtained at 670 nm wavelength, it happens to be the starting wavelength of the OPO laser in the NIR range. It was found that the laser output was unstable at the initial excitation wavelength. Hence, in this work, we have used 675 nm wavelength, for both MCES and experimental work.

Figure 11: (a) Optical spectrum of methylene blue in the NIR range, (b) Photoacoustic spectrum of the methylene blue in the NIR wavelength range. The PA signals were normalized by the laser energy at each wavelength.

Monte Carlo simulations were carried out to optimize the light delivery to the SLN as described. The mean (standard deviation) spot size from each fiber bundle is 4.4 (0.58) cm and 0.6 (0.001) cm along X and Y axis, respectively. Fig. 12(a) shows the absorbance in sphere for variation in FPD (study 1). When the illumination angle is 89° ($\sim 90^\circ$, which implies photons are parallel to the tissue surface), negligible number of photons enters the simulation medium (yellow star). The maximum absorption was at 30° (blue circle), 45° (brown inverted triangle), and 60° (blue star) for FPD of 1 cm, 2 cm, 3 cm, respectively. When none of the photons were blocked by the transducer, with increase in the FPD, higher angle of illumination was needed for maximum absorption. Also note that with increase in FPD the maximum absorption decreases. Hence, to increase the signal (absorption) one should try to illuminate closer to the object with lower illumination angle. In practice getting very low FPD is not possible as the ultrasound probe is placed between the fiber-bundles.

Fig. 12 (b) shows the results from study 2. It was observed that for lower FPD (1 cm), illumination at 30° did not produce maximum absorption as seen in study 1. This was because of the partial blockage of photons hitting the probe and those photons did not reach the imaging area. At this FPD, illumination at angles higher than 30° gave nil absorption since none of the photons (or very few photons in practice could reach) reached the simulation medium (tissue). For FPD of 2 cm, partial light from 60° illumination was blocked and all photons were blocked for illumination of 75° (brown right sided triangle) and 89° . When FPD was increased to 3 cm, illumination at 0° to 60° was unaffected, whereas light for 75° illumination was partially blocked. Since the absorption for 75° and 89° was negligible, these two angles were not considered for further studies. FPD of 1 cm is ideal for getting maximum light reaching the target object (SLN). However, due to the size of the probe, the optical fiber casing and the

fiber holder design, FPD of 1 cm was unrealistic and infeasible. Therefore FPD of 2 cm was chosen for study 3.

Figure 12: Simulation results. (a) Study 1 - Absorbance in sphere for angle of illumination varying from 0°, 15°, 30°, 45°, 60°, 75°, and 89° when fiber-to-probe distance (FPD) was increased from 1 cm to 3 cm (fiber-to-tissue distance was fixed 1 cm). (b) Study 2 - absorbance in sphere for angle of illumination varying from 0°, 15°, 30°, 45°, 60°, 75°, and 89° when FPD was increased from 1 cm to 3 cm with the photons which fall on the transducers being blocked. (c) Study 3 - FPD was 2 cm, FTD was varied from 1 cm to 4 cm for angles 0°, 5°, 10°, 15°, 20°, 30°, 45°, and 60°. (d) Study 4 - Absorbance in SLN for spheres at depths 0.5 cm to 3 cm in steps of 0.5 cm when FTD was kept at 3 cm, FPD for 0° was 1.9 cm, 1.7 cm for 5° and 10°, and 1.8 cm for 15°. The angles of illumination were 0°, 5°, 10°, and 15°. (e-h) The absorbance maps of sphere at depth 0.5 cm for illumination angles 0°, 5°, 10°, and 15°.

Fig. 12(c) shows the result of study 3, where FPD was fixed at 2 cm, sphere was at 1.5 cm under the tissue surface, and the FTD was varied from 1 cm to 4 cm for illumination angle of 0° (blue plus), 5° (brown cross), 10° (yellow square), 15° (purple diamond), 20° (green triangle), 30° (blue circle), 45° (brown inverted triangle), and 60° (blue star). With the increase in the fiber to tissue distance (FTD), for higher angle of illumination, amount of photons blocked increased. FTD was varied from 1 cm to 4 cm, maintaining a FPD of 2 cm (distance between the probe outer surface and center of fiber bundle is

1.5 cm). Theoretically $[\tan^{-1}(1.5/FTD)]$, the illumination photons above angles 56° , 37° , 27° and 21° were partially or completely blocked at FTD of 1 cm, 2 cm, 3 cm, and 4 cm, respectively. Hence, for FTD of 1 cm, 2 cm, 3 cm, and 4 cm, illumination angle of 45° , 30° , 15° , and 10° gave maximum absorption. Since, the flexibility of the fiber bundle due to its heavy metal casing confined the bending angle to 15° , to maximize the light absorbance inside the sphere we chose to use FTD of 3 cm for further study.

Study 4 was done to see the effect of different illumination angles at different depths of the SLN. This study was also experimentally validated for SLN (spheres) at different depths. Hence, FPD was chosen within 1.7 cm to 1.9 cm (which is close to 2 cm) depending on the bundle holder fabricated. Due to the additional limitation of the flexibility of the fiber bundle, maximum possible illumination angle was 15° . At FTD of 3 cm, we observed maximum absorption at this angle [Fig. 12(c)]. Hence, FTD of 3 cm was chosen for both simulations and experiments in this study. Fig.12 (d) shows the absorbance plot (spheres placed at depth 0.5 cm to 3 cm) for illumination angles 0° (blue plus), 5° (brown cross), 10° (yellow square), and 15° (purple diamond). For spheres at depths (0.5 cm and 1 cm) illumination of 15° gave the maximum absorption. 10° gave the maximum absorption for other depths. Trend in the plots reveal that the angle of launch plays an important role for delivering light from different distances between the fiber-probe and fiber-tissue. With increase in the angle of illumination, propagation of photons towards the center of the geometry (closer to the origin) increases the light absorbance in the sphere. The variation across maximum and minimum absorption in sphere across angles 0° to 15° in steps of 5° was 110%, 86%, 66%, 52%, 42%, and 36% for 0.5 cm, 1 cm, 1.5 cm, 2 cm, 2.5 cm, and 3 cm sphere depth, respectively. Fig. 12(e-h) shows the light absorption maps in the SLN at depth 0.5 cm. 320 million photons were simulated for obtaining the smooth absorption maps.

Figure 13: Experimental results. (a) Plot showing the SNR of the PA images for various depths of the SLN (0.5 cm, 1 cm, 1.5 cm, 2 cm, and 2.5 cm) and for different light launching angles (0°, 5°, 10°, and 15°). Fiber to tissue distance was kept at 3 cm. (b) Screenshot captured from the Ecube display of the combined ultrasound and PA imaging [the SLN (1 cm diameter sphere filled with methylene blue) was placed at a depth of 0.5 cm under the chicken tissue].

Lastly, experiments were done to validate the simulation results from study 4. Probe holders were designed to hold the fiber bundle at angles 0°, 5°, 10°, and 15°. Due to the limited length and limited flexibility of the fiber bundle inclination, more than 15° for light delivery was not feasible. Hence, the maximum angle of light delivery was experimentally restricted to 15°. Phantom experiments were carried with all the 4 different probe holders for various SLN depths of 0.5 cm, 1 cm, 1.5 cm, 2 cm, and 2.5 cm. The fiber to tissue distance was kept at 3 cm. Although in simulation 3 cm deep imaging was done, it was not done experimentally as the PA signals were very weak for this depth (simulation showed that the sphere placed at 3 cm absorbed only one-tenth of the photons in comparison to a sphere placed at 0.5 cm). Fig. 13(a) shows the signal-to-noise ratio obtained from the beam-formed PA images (after Hilbert transformation) of the SLN phantom using the clinical PA imaging system. The SNR

was defined as the amplitude of the PA signal from the SLN divided by the standard deviation of the background noise, $SNR = \frac{V}{n}$, here V is the PA signal amplitude from SLN, and n is the standard deviation of the background noise. To obtain the signal from the SLN, a rectangular region was chosen manually in the area where the SLN phantom was located. The average of the top ten pixels in the region was taken as the signal value (V). To calculate noise (n), a rectangular area was taken near the SLN and the standard deviation was calculated. Using the above two values, SNR was calculated. The size of the region of interest (ROI) used to calculate the SNR (both signal and noise) was the same for all the images. No masking was used for the selection of the target feature. The SNR is plotted with respect to the depth of the sphere. Multiple plots in the graph represent the different angles of light delivery (or different probe holder). No acoustic attenuation correction was performed for the experimental photoacoustic results.

Trend in the SNR confirms experimentally that probe holder design is important depending on the imaging depths and target locations. At most of the depths the SNR obtained at 0° was the lowest. At shallower imaging depths, the variation in SNR is more than 100%. At an imaging depth of 2.5 cm, the SNR values do not vary significantly which is as expected. With increase in depth, the SNR drops irrespective of the angle of launch. From Fig. 13 (a), it is observed that the maximum SNR was obtained at an angle of 15° for 0.5 cm, and for other depths 10° gives the maximum SNR, which closely matches with our simulation results. Fig. 13(b) shows the screenshot captured from the Ecube display monitor of the combined ultrasound and PA imaging of the SLN at 0.5 cm. The grayscale image represents the ultrasound image and the coloured overlap represents the PA. This image is shown to confirm the position of the sphere. For representative purpose, the B-scan PA images of the sphere placed at 0.5 cm depth for different angles of light delivery is also shown. To reduce the effect

of energy variation in the laser, the Hilbert transformed PA images shown are averaged from 25 frames (Beam-formed images). Figs. 13(c-f) show the PA images at 0.5 cm depth. Comparing the absorption maps obtained from the simulation and the PA image, SNR plot obtained from the experimental results, it is concluded that the simulation data is a close match to the experimental outcome, as the results obtained from both follows a similar trend. It is to be noted that the simulation and the experimental results will not match 100% as there are many factors that cannot be controlled in the experimental scenario including experimental error. Also, we are comparing the absorbance measured in simulations versus the SNR from the PA images, which can lead to minor deviations in the results. However, simulation data can be used as a precursor since the general trend is similar in both cases. Due to practical difficulties light delivery angle more than 15° was not done experimentally. For all the depths, for most of the values the simulation's absorption trend coincides with the trend of the PA image SNR obtained from experimental data. Hence, Monte Carlo simulation with embedded objects can be used as a precursor to design the probe for various experimental needs. This highly reduces the trial and error method in choosing the angle of light delivery when the object is known to be located at a certain depth or reduces the risk of using just one angle of light delivery irrespective of how deep the object is. In clinical application, this will be very useful by reducing the amount of exposure to laser light by eliminating the necessity to experiment with different angles of light delivery in order to obtain the best results. Also, if the depth of imaging is known it greatly reduces the actual imaging time on the patient.

There are several factors like the angle of illumination, the distance between the transducer and the fiber tip, the distance between the fiber and the skin surface etc., which can affect the PA signal. Although, we have considered all these factors in

simulation studies, experimental validation is provided only for different light illumination angle with respect to depth at a fixed fiber tissue distance owing to practical limitations of the fiber bundle. The kind of imaging setup (imaging area, tissue properties etc.) one is interested will also play a key role. For example, here we have used SLN imaging scenario. For a different application, there will be different types of tissues, the target object structure could also be different. In this work we have considered the sentinel lymph node as a spherical model. We have used this only as a model to demonstrate that simulations can be used as a precursor to optimize clinical imaging. Another factor is the illumination light wavelength. In this particular problem since methylene blue is used as contrast agent for the SLN mapping, the excitation wavelength used is 675 nm, since MB has peak absorption close to this wavelength. So to conclude, the user may need to do the appropriate simulations with all these factors taken into consideration before designing the probe holder for a particular problem they have in hand. Another challenge is to have a single probe holder where the angle of illumination can be controlled. At present, we have several separate probe holders and for each angle of illumination we need to change the probe holder. Ideally one probe holder with adjustable angle will be better for *in vivo* imaging setup.

3.4 Conclusion

Monte Carlo simulations were done to study the effect of the light launching angles on the PA signal amplitude for a handheld photoacoustic probe. Different simulation scenarios were considered for the different parameters affecting the imaging. Experimental validation was provided for the different illumination angles at various SLN depths for a given fiber to tissue and fiber to probe distances. It is shown from simulations that different illumination angle is needed to obtain maximum absorbance in SLN. Accordingly, several 3D printed probe holders were designed and PA imaging

was done using the clinical ultrasound platform. It was confirmed through experiments that the angle of light delivery plays an important role with respect to the depth of imaging at a given FTD. Using this system sentinel lymph node imaging and urinary bladder imaging is shown in small animals in the following chapter.

Chapter 4

Applications of clinical PA imaging

4.1 Non-invasive sentinel lymph node mapping and needle guidance³

4.1.1 Introduction:

Invasive breast tumor is one of the leading causes of cancer deaths among women [92]. Diagnosing and staging it early is important for deciding treatment strategies, which plays an important role in the prognosis of the disease. In most cases of invasive breast tumors, surgical removal of primary breast tumor and axillary lymph node dissections (ALND) are common practice. There are lot of side effects associated with ALND including arm numbness, arm weakness, upper-extremity lymphedema, impaired shoulder mobility, and infections in breast, chest or arm [93]. Therefore, a more accurate, less complex and less invasive technique of sentinel lymph node biopsy was developed as an alternate. For node-negative breast cancer, SLNB has become the gold standard for initial screening [94, 95]. Lymph nodes are small oval-shaped organs that are present all through the body as part of the lymphatic system. They are connected via lymph vessels [96]. SLNB works on the assumption that sentinel lymph node will be the most probable primary site for metastases. Although this technique is more accurate and less complicated than ALND, its sensitivity is less than 95% [97, 98].

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K. Sivasubramanian, V. Periyasamy, and M. Pramanik, “Hand-held clinical photoacoustic imaging system for real-time non-invasive small animal imaging,” *Journal of Visualized Experiments* 128, e56649 (2017).

Also, there are side effects associated with SLNB procedure, including lymphedema, seroma formation, sensory nerve injury and limitation in the range of motion [99]. Therefore, it is very important to find non-invasive methods of identifying and staging the axilla without involvement of surgery (using fine needle aspiration biopsy) to overcome these limitations.

Ultrasonography is an imaging technique that is commonly used to examine the axilla for pre-surgical assessment. US helps to visualize the size, shape, contour, texture, and other properties of lymph nodes which will differ if cancer cells have metastasized in comparison to normal lymph nodes [100, 101]. However, the accuracy of this technique is low, and it does not have the ability to identify and differentiate SLNs from other lymph nodes. Differentiating SLN is critical during the surgery to remove SLNs only and leave the other lymph nodes intact. The possibility of false positive and false negative detections is also very high with this method. Therefore, US imaging cannot be used singularly for identifying, mapping, and staging of the breast cancer based on metastasis to SLNs.

Conventionally SLNB procedure involves injecting of methylene blue dye (or other dyes) and/or radioactive tracers to trace the lymphatic system and help in identifying the sentinel lymph node [98, 102, 103]. When both are used together the accuracy is improved [104]. The radioactive tracer has to be injected hours before the SLNB procedure, and methylene blue can be injected on the operating table itself as it moves quickly through the lymphatic system. After injection the region is cut open by making a small surgical incision. The surgeon then inspects the area visually to identify the blue coloured nodes in case of methylene blue to identify the SLN. To identify the SLN with radioactive tracer a Geiger counter is used. Few SLNs are then removed from the patient and subjected to histopathological studies to identify the presence or absence of cancer

cells. Negative SLNB result indicates that cancer has not metastasized to nearby lymph nodes or other organs and a positive SLNB result indicates that cancer has metastasized to the sentinel lymph node and it is possible that it may be present in other nearby lymph nodes and, other organs. Although, this procedure is less invasive and complications associated are less severe than ALND, a non-invasive way of doing SLNB is very essential and highly beneficial for patients.

A lot of work has been previously reported for SLN imaging and mapping in small animals as well as in humans using different contrast agents [59, 66, 105, 106]. However, most of the reported photoacoustic systems cannot be used for translated to clinical scenario due to many factors. Some of which are: 1) immovable system design, hence is not convenient for clinical use, 2) use of non-clinical ultrasound transducers (as well as ultrasound machine) for data acquisition [51, 107]. Clinical translation with such transducers (system) is not possible; therefore use of clinical ultrasound imaging system is more preferred. Also, most of the reported work using clinical ultrasound systems is with systems that are not commercially available yet [39, 59, 63]. For PAI to be integrated with clinical ultrasound system, access to raw channel data is required, which was previously available in research based ultrasound systems only or custom made clinical ultrasound machine (not available to any researcher to purchase). Only recently, clinical ultrasound systems with access to raw channel data is made available commercially [37]. Another problem that has not been addressed sufficiently thus far is how SLNB surgery can be avoided by performing non-invasive needle guidance for fine needle aspiration biopsy. This will eliminate the complications associated with SLNB surgery. Here, a clinical ultrasound system for combined US and PA imaging was used. For ease of use in clinical setup, a custom made handheld holder for housing optical fiber and ultrasound transducer was fabricated. Two different dyes (methylene

blue and indocyanine green) were used for identifying and mapping SLNs. Additionally, to eliminate the complications associated with the SLNB surgery, non-invasive real time needle tracking and guidance to remove the SLN tissue is also demonstrated.

4.1.2 Materials and Methods

4.1.2.1 Photoacoustic and optical absorbance spectrum of the dyes used

Methylene blue is a clinically approved and a widely used contrast agent for sentinel lymph node imaging [63, 108]. Indocyanine green has also been explored for SLN imaging [64]. Therefore, these two dyes were used for all experiments in this work. From literature MB has an absorption peak around 668 nm [91], and absorption of ICG is highly concentration dependent [51]. For experiments, 1000 μ M concentration of ICG was chosen. The reported absorption peak for this concentration is 698 nm. In order to find an optimum wavelength to work, optical absorbance and PA spectrum of MB and ICG in the near infrared (NIR) wavelength range was recorded. To record the optical spectrum of the dyes, UV-visible spectrophotometer (Spectramax M5) was used. The wavelength was varied from 675 nm to 800 nm in steps of 5 nm. The concentration of MB and ICG used was 0.01 mg/mL and 0.00076 mg/mL, respectively. For animal blood photoacoustic spectrum, rat blood was used. For recording the PA spectrum, 10 mg/mL of MB (Sterop, Belgium) or ICG of 1000 μ M concentration (I2633, Sigma Aldrich) or rat blood was injected into a transparent low density polyethylene tube of diameter 1.02 mm and placed in water bath. A single element ultrasound transducer (2.25 MHz center frequency, V306-SU, Olympus-NDT) was used for PA signal acquisition. The signals were first amplified, and then band pass filtered by ultrasound signal receiver (R/A/F) unit (5072PR, Olympus-NDT), and then digitized and stored in a desktop with data acquisition (DAQ) card (compuscope 4227,

GaGe). The OPO laser (Continuum, Surelite OPO) used here has a minimum wavelength of 670 nm. However, there is a lot of fluctuation in the starting wavelength. Therefore, laser was varied from 675 nm to 800 nm wavelength with a 5 nm step size similar to optical spectrum. Each of the obtained PA signals was averaged 10 times. The peak-to-peak PA signal intensity is plotted against the wavelength. The PA signals were normalized with the laser power.

4.1.2.2 Clinical handheld photoacoustic imaging system

The imaging system is the same as described in section 3.2.4. For imaging experiments the laser was tuned to 675 nm for MB (10 mg/mL) and 690 nm for ICG (1000 μ M). The light from the laser was coupled to the sample with a bifurcated optical fiber bundle (Ceramoptec GmbH, Germany) with 1600 optical fibers. Each fiber has a core diameter of 185 μ m (200 μ m with cladding) with a numerical aperture of 0.22. The two output ends of the fiber bundle were fixed in the custom made (3D printed) probe holder along with the linear array ultrasound transducer to make a handheld system. The angle of illumination of light for this holder was 15°. The different probe holder parameters were optimized for SLN imaging using Monte Carlo simulations for light delivery and PA imaging with SLN mimicking phantoms [109]. The energy loss due to fiber coupling was approximately 40%. The light energy on the sample surface was ~25 mJ per pulse at 675 nm. The fluence on the surface of the sample calculated to be ~8.34 mJ/cm² (total area of illumination from both ends together is ~3 cm²). Thus, the fluence was well within the American National Standards Institute safety limit of 20 mJ/cm² [76].

4.1.2.3 Animal preparation

Animal experiments were performed in accordance with the approved guidelines and regulations, and were approved by the Institutional Animal Care and Use committee of Nanyang Technological University, Singapore (Animal Protocol Number ARF-

SBS/NIE-A0263). For experiments, 6 healthy adult female Sprague dawley rats of weight 225 ± 25 gm (aged 8 - 10 weeks) were procured from InVivos Pte. Ltd., Singapore. Two animals (both sides of the rat was imaged) each for imaging with saline, MB and ICG were used. Firstly, before performing *in vivo* imaging the rats were anesthetized. Anaesthesia mixture contains Ketamine and Xylazine (dosage of 85 mg/kg and 15 mg/kg, respectively). An intraperitoneal injection of 0.2 ml per 100 g of the rat body weight was given. Subsequently, the animal was prepared for imaging by removing hair in the region of interest using commercially available hair removal cream. A custom made nose cone covered with a breathing mask covering the mouth and nose of the animal was used to deliver the anaesthesia mixture. During experiments, animal was maintained under anaesthesia all through with the help of isoflurane gas (Medical Plus Pte Ltd, Singapore). 0.75% of isoflurane was administered along with oxygen (1.2 L/min). A pulse oximeter (Medtronic, PM10N with veterinary sensor) was used to monitor the heart rate and peripheral oxygen saturation during experiments. The animal was placed sideways and combined PA and US images were recorded with the handheld probe before and after dye injection. After acquisition of the PA images, the animals were euthanized with a pentobarbital overdose. Finally the SLNs were dissected out for study.

4.1.2.4 Sentinel lymph node imaging in rats

The schematic representation of the imaging system is shown in Fig. 14. To image SLN a custom made handheld probe holder housing the ultrasound transducer and optical fiber for light delivery was used. The probe holder design was optimized for SLN imaging in our previous work [109]. The parameters used for imaging are fiber to probe distance of approximately 2 cm, fiber to tissue distance of 1 cm, and angle of light illumination of 15° . In rats SLN is usually present just below the skin surface. To

emulate a more realistic human SLN imaging scenario a 0.5 cm chicken breast tissue (CBT) was placed on top of the rat. Ultrasound gel was spread on top of the tissue for better ultrasound coupling. Using the handheld PA imaging system the sentinel lymph nodes in rats were imaged before and after the injection of the dye (saline or MB or ICG). Saline was used as a control study. Control image of the SLN was taken before the dye injection. 0.2 mL of the dye (MB or ICG) was injected in the forepaw of the imaging side of the animal. The forepaw of the animal was massaged for 5 min to promote the movement of the dye in to the lymph nodes. Following which the lymph nodes were imaged and identified using the handheld PA probe. For confirmatory studies wavelength was varied from 670 nm to 800 nm at a speed of 10 nm/s while holding the probe at a fixed location. The spectroscopic data from the SLN was later compared to the spectroscopic data obtained *in vitro*. Also, to cover all realistic scenarios for human SLN imaging, up to 1.5 cm deep imaging was done with the help of additional chicken tissue slices.

Figure 14: Schematic representation of the Photoacoustic imaging system with clinical ultrasound platform. OPO - optical parametric oscillator, OF - optical fiber bundle, FH – 3D printed fiber holder, USM - clinical ultrasound machine, UST- ultrasound transducer. The fiber holder houses the two output optical fiber bundle for illumination of tissue and ultrasound transducer for signal acquisition.

4.1.2.5 Needle tracking

Non-invasive imaging and mapping of sentinel lymph nodes is the preliminary step in staging of breast cancer. The imaging region needs to be cut open to remove the lymph nodes for biopsy, which is a painful procedure and has lot of complications associated with it. We have shown here real time needle guidance (minimally invasive) to remove tissue from SLN for further biopsy. This is done at 675 nm and 690 nm where there is maximum optical absorption for MB and ICG, respectively. After identification of sentinel lymph nodes in rats, the combined handheld PA probe holder was held steady in that position. A 23G needle (BD PrecisionGlide Needle) of dimensions 0.6 mm $\times$ 32 mm was slowly inserted into the animal through the chicken tissue parallel to the longer side of the ultrasound transducer while tracking it in real time on the E-CUBE's monitor. When the needle reaches the sentinel lymph node some tissue can be extracted out on which further histopathological studies can be done for staging the tumor, if any is present.

4.1.3 Results and Discussion

MB is a *Food and Drug Administration* (FDA) approved dye and is widely used clinically for SLNB. Therefore, MB (due to its strong light absorption) has been used for noninvasively imaging sentinel lymph node with photoacoustic imaging extensively [39, 63, 108]. ICG is another dye that is being explored for SLN imaging [51, 64]. Both these dyes are used as PA contrast agents in this study. In order to determine the most suitable wavelength in the NIR region for both the dyes, UV-visible and PA spectrum was recorded *in vitro*. To compare with the feasible experimental conditions, UV-visible spectrum is recorded in the wavelength range of 675 to 800 nm. The UV-visible spectrum of methylene blue and ICG is shown in Figs. 15(a) and 15(c), respectively.

The PA spectrum of MB and ICG is shown in Figs. 15(b) and 15(d), respectively. It is to be noted that the maximum peak-to-peak PA signal intensity is at 675 nm and 690 nm for MB and ICG, respectively and hence, chosen for experiments. It can be observed that the PA spectrum of MB and ICG is in correlation with their respective optical absorption. The PA spectrum of rat blood is shown in Fig. 15 (e).

Figure 15: (a) Optical spectrum of methylene blue (MB) in the near infrared (NIR) range, (b) photoacoustic spectrum of the MB in the NIR wavelength range, (c) optical spectrum of indocyanine green (ICG) in the NIR range, (d) photoacoustic spectrum of the ICG in the NIR wavelength range, (e) photoacoustic spectrum of rat blood in the NIR wavelength range. The PA signals were normalized by the laser energy at each wavelength. 675 nm to 800 nm wavelength range with 5 nm step was used for both optical and photoacoustic spectra.

After determining the optimum wavelength for imaging, animal experiments were performed. Saline was used as control as it does not have light absorption in the NIR range and does not produce any contrast for photoacoustic imaging. Fig. 16(a) shows the photograph of the shaved regions of a rat for SLN imaging. Black dotted line shows

roughly the plane across which B-scan ultrasound as well as photoacoustic imaging were done. All combined PA and US images shown are screenshots taken from the E-CUBE monitor. Fig. 16(b) and 16(c) shows the combined US and PA images before and after injection of saline. It is to be noted that we can identify the lymph nodes with the help of ultrasound only, but the contrast is not very high and it needs a trained eye to identify them. Even though ultrasound imaging can identify the lymph node, it cannot differentiate the sentinel one. As expected, there is no PA signal before and after injection of saline, thus, saline cannot help mapping the SLN with PA imaging. Fig. 16(d) shows the photograph of the sentinel lymph node after skin removal after imaging was finished. Fig. 16(e) shows the photograph of the excised SLN from the rat. It can be noted from the image that SLN is not coloured, but it is bulged indicating that it is completely perfused with saline. Fig. 16(f) and 16(g) show the combined US and PA images before and after injection of MB, respectively. The gray colours represent the US image and the coloured part shows the PA images. It can be observed that there is no PA signal before injection of MB in the SLN area. But after injection of MB, there is an increased PA signal in the SLN, and therefore, it is visible in Fig. 16(g). Fig. 16(h) shows the photograph of the SLN with MB after skin removal. The blue coloured stains helps in easy identification with naked eyes. The image also shows the lymph vessels filled with the MB. Fig. 16(i) shows the photograph of the excised SLN completely filled with MB. From this image we can compare and confirm the size of the SLN with the combined US and PA image. Similarly, Fig. 16(j) and 16(k) shows the combined US and PA images before and after injection of ICG. It is evident from the images that there is no PA signal from the SLN before injection of ICG, but after injection the PA signal is very prominent. Fig. 16(l) shows the photograph of the SLN with ICG after removing the skin. The SLN is not obviously stained visually as opposed to MB (since

ICG solution is colourless or light greenish in appearance). Nevertheless, they are perfused with ICG solution which helps in differentiating them from other normal lymph nodes. Fig. 16(m) shows the photograph of the excised SLN completely filled with ICG. As stated earlier, from the image we can compare and confirm the size and location of SLN with the combined US and PA image.

Figure 16: (a) photographs of the shaved imaging region of the rat for imaging with saline, (b) combined US and PA image before saline injection, (c) combined US and PA image after saline injection, (d) Photograph of SLN after small incision on skin, (e) Photograph of excised SLN, (f) combined US and PA image before MB injection, (g) combined US and PA image after MB injection, (h) Photograph of SLN after small incision on skin, (i) Photograph of excised SLN, (j) combined US and PA image before ICG injection, (k) combined US and PA image after ICG injection, (l) Photograph of SLN after small incision on skin, (m) Photograph of excised SLN. 675 nm wavelength was used for PA imaging with saline and MB, 690 nm wavelength was used for imaging with ICG.

The optical absorption of MB is wavelength dependent, whereas for ICG it is concentration dependent as well. MB has a peak around 670 nm and the peak is quite

sharp. In comparison ICG has broader absorption profile in the NIR region. It is expected that the wavelength dependent absorption will reflect in the *in vivo* SLN imaging. For confirmatory studies spectral analysis was used by varying the wavelength from 670 nm and 800 nm and imaged in real time on the E-CUBE's monitor. This helped in providing evidence that the PA signal was from SLN filled with the dye only. The combined PA and ultrasound imaging as the laser wavelength was scanned is shown in Fig 17(a-c) at 670, 700, and 800 nm respectively after MB injection. Fig. 17(d-f) shows the combined US-PA images after ICG injection at 670, 700, and 800 nm respectively. In case of MB, as the absorption peak is sharp, the PA signal disappears quickly as the laser wavelength is scanned. Confirming the PA signal is from the SLN filled with MB. Similarly, in case of ICG, the PA signal decreases rather slowly, but does not disappear completely unlike the case of MB, as the absorption peak of ICG is much wider in the chosen wavelength range. Also, while varying the wavelength from 670 nm to 800 nm, the PA signals were recorded and saved as beam formed data. From the saved data, the signal-to-noise ratio (SNR) from the SLN area is calculated at different wavelengths and plotted as PA spectrum of *in vivo* data. Additionally, for comparison PA spectrum (in terms of SNR) is obtained from the nearby blood vessel at various wavelengths. All the spectrums are normalized with the power of laser at different wavelengths. Figs. 18(a) and 18(b) shows the PA spectrum from SLN for MB and ICG, respectively. Fig. 18(c) shows the PA spectrum obtained from the blood vessel from a nearby region. It can be noted that the trend in the PA spectrum from the SLN in rat matches with the PA spectrum obtained with the respective dyes *in vitro* [Figs. 15(b) and 15(d)]. While the PA spectrum of MB and ICG is decreasing, it can be noted that there is not much change in the PA spectrum of blood vessel, which matches well with the *in vitro* PA spectrum of blood [Fig. 15(e)].

Figure 17: (a-c) combined US-PA images of the SLN at 670, 700, and 800 nm respectively after injection of MB; (d-f) same as (a-c) after injection of ICG.

Figure 18: (a) PA spectrum obtained from the *in vivo* PA images (in terms of SNR) of the rat SLN with methylene blue, (b) spectrum with indocyanine green, (c) spectrum of the rat blood vessel. SNR was calculated for different wavelengths and normalised with the power of the laser.

To simulate SLN imaging in humans, chicken tissue was placed on the top of the skin surface of the rats, thus the SLN lies 1-2 cm below the chicken tissue surface. The

thickness of the chicken breast tissue was increased in steps of 0.5 cm on top of the rat to simulate depth imaging. The thickness of the chicken breast tissue was increased in steps of 0.5 cm on top of the rat to simulate depth imaging. Imaging was done at 675 nm and 690 nm for MB and ICG, respectively. The combined US and PA images of SLN filled with MB at different depths of 0.5 cm, 1 cm, and 1.5 cm is shown in Fig. 19(a), 19(b) and 19(c), respectively. Similarly, combined US and PA images of SLN with ICG at various depths of 0.5 cm, 1 cm, and 1.5 cm is shown in Fig. 19(d), 19(e) and 19(f), respectively. It can be noticed from the images that the SLN is clearly visible even at higher depths for both the dyes. Additionally, SNR was also calculated from the SLN at different depths. SNR was defined as the amplitude of the PA signal from the SLN divided by the standard deviation of the background noise, $SNR = \frac{V}{n}$, here V is the PA signal amplitude from SLN, and n is the standard deviation of the background noise. To get the signal, a small rectangular area was selected manually around the SLN location. The average of the top five pixels was taken as the signal (V). To determine noise (n), a rectangular region was chosen near the SLN and the standard deviation was calculated. Using the above two values, SNR was calculated. The calculated SNR for MB at depths of 0.5 cm, 1 cm, and 1.5 cm are 33.40, 57.9, and 21.02, respectively. The SNR for ICG was calculated to be 11.17, 5.6, and 2.15 for depths of 0.5 cm, 1 cm, and 1.5 cm, respectively. SNR at 1 cm is highest in case of MB which is consistent with our simulation and phantom results published earlier, due to the light delivery angle [109]. With ICG the SNR decreases with increase in depth, which is as expected. From the figures, it can be concluded that both ICG and MB are viable and effective contrast agents for SLN imaging in a clinical scenario.

Figure 19: Combined PA and US images of (a) SLN filled with MB at 0.5 cm depth, (b) SLN filled with MB at 1 cm depth, (c) SLN filled with MB at 1.5 cm depth, (d) SLN filled with ICG at 0.5 cm depth, (e) SLN filled with ICG at 1 cm depth, and (f) SLN filled with ICG at 1.5 cm depth. CBT- Chicken breast tissue. The imaging depth were varied by stacking layers of CBT on top of the rat.

Non-invasive identification together with fine needle aspiration biopsy (FNAB) of SLN will reduce complications associated with SLNB surgery. Ultrasonography is the most commonly used technique for needle guidance, catheter placement, etc. till now [110]. But, the contrast provided by ultrasound is not great to visualize needle going inside tissue. Non-invasive, real time needle guidance for biopsy of SLN with photoacoustic imaging is also shown here. As soon as SLN was identified in the rat by PA imaging, the probe holder was held fixed in the position. A 23G needle (0.6 mm diameter) was inserted parallel to the longer side of the ultrasound transducer and guided to the SLN by real-time monitoring of the needle on the E-CUBE's screen. Fig. 20(a) shows the image of US guided needle biopsy by the needle into the SLN. Fig. 20(b) and 21(c) show the combined US and PA images of the guided needle biopsy of the SLN. For comparison pure US guided imaging was also performed. The contrast provided by US was quite poor and one needs a trained eye to follow the needle and guide it properly [Fig. 20(a)]. On the other hand, with PA imaging, the contrast from the needle is very

high and can be easily monitored *in vivo* [Fig. 20(b) and 20(c)]. Once, the needle reaches the SLN a small portion of the SLN tissue can be taken out for histological examination. It is to be noted that the artefact above the needle is due to the reconstruction and post-processing done by the system. The needle can be differentiated from the artifact based on the increased signal intensity in comparison to the artifact. Additionally, the signal from the needle is a single straight line across depth while the signal from the artifact is divergent with depth. These factors will help in identifying and differentiating the needle from the artifact.

Figure 20: (a) US image showing the needle tracking as indicated by the yellow arrow, (b) combined US and PA image showing the needle reaching the sentinel lymph node filled with methylene blue, (c) combined US and PA image the needle reaching the sentinel lymph node filled with indocyanine green. CBT- Chicken breast tissue.

The cost associated with screening, diagnosis and treatment of breast cancer is very high, and the number of cases reported is increasing every year. Therefore, a cost effective, fast, less complicated, and more efficient imaging and guidance system is much needed. Combined US and PA imaging is one such technique which can be used for effective screening, diagnosis and staging of breast cancer. There is lot of research going on to explore the potential of this technique. This is easily feasible with FDA approved contrast agents such as MB, which is already being used routinely for SLNB procedure. The number of false positives and false negatives can also be decreased significantly using dual modality imaging. Since, it is a non-invasive procedure the

complications and pain associated with surgery will be eliminated for patients. In this work, we have demonstrated using a dual modal photoacoustic and ultrasound system (based on a clinical ultrasound platform) SLN can be identified using both MB as well as ICG. Moreover, under PA imaging the needle guidance in real time for FNAB in place of SLNB surgery can also be improved. There are few challenges associated with this technique. Firstly, the lasers currently used for PA imaging are very bulky, and portable high energy pulsed lasers although available, are quite expensive. Secondly, the integration of light delivery with the ultrasound probe is also a challenging task with high laser light coupling efficiency. Lastly, MB has an absorption peak ~665 nm wavelength. Currently, availability of lasers at this wavelength is limited. Therefore, most researchers are using 670-675 nm lasers. Although the difference in wavelength is very small, but there is a significant difference in light absorption (since MB absorption peak is very sharp).

4.1.4 Conclusion

In this part, we have demonstrated that a commercial clinical ultrasound system can be used for combined US and PA imaging of SLN in small animals (rat). To provide imaging contrast, MB and ICG were used as they have high optical absorption in the NIR wavelength region. Moreover, MB is already been used in clinic for SLNB, therefore, it will not affect the clinical workflow. Spectral studies were done to confirm that the SLN images obtained are due to the lymph node filled with the dye. SLN imaging at varying depths of up to 1.5 cm was done to make it suitable for clinical scenario. Additionally, non-invasive needle guidance for FNAB was performed by tracking the needle in real time under photoacoustic imaging. This will be very useful in eliminating the necessity for SLNB surgery and thereby avoiding the complications associated with it.

4.2 Rat urinary bladder and its applications⁴

4.2.1 Introduction

Urinary bladder imaging is essential to monitor disorders such as urinary incontinence, vesicoureteral reflux, cystitis, glomerulation, bladder cancer, etc. [111-113]. Severity of the diseases varies from causing minor discomfort to being fatal. Urinary incontinence is a condition when the person suffers from involuntary leakage of urine from the bladder. It can occur due to loss of control or failed sphincter muscle. This problem usually occurs with aging. Vesicoureteral reflux (VUR) is the reverse flow of urine from the bladder to the kidney through the ureters. Urine usually flows from the kidney to the bladder through the ureters. The sphincter valve present at the junction of the ureters and bladder prevents the reverse flow. If reflux occurs, bacteria present in the bladder can move to the kidney causing further infection, scarring etc. The reflux can occur while voiding urine or at rest [114]. Cystitis is the condition where bladder is infected due to bacteria [115]. If not treated on time, this can lead to further complications along with discomfort to the patient. Glomerulation is caused by bladder hemorrhages [116]. Glomerulation and cystitis are highly related. While most of the urinary tract infections occur more commonly in women, bladder cancer occurs more frequently in men. The cancer begins on the wall of the bladder and eventually spreads, which can be fatal to the patient. Therefore, imaging the urinary tract (ureter, bladder and urethra) is very important [111, 117].

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Some of the commonly used imaging techniques for bladder imaging include cystography, cystourethroscopy, ultrasound imaging, optical imaging, etc. Cystography is the gold standard for bladder imaging to evaluate bladder cancer, VUR, bladder polyps, hydronephrosis (inflammation of kidney due to water retention), etc. In cystography, a radio opaque contrast is injected and X-ray imaging is used to obtain images of the bladder before, during, and after voiding to identify bladder related diseases [118, 119]. The sensitivity of the technique is not very high and also ionizing radiations are used for imaging. This raises the necessity for using safer and highly sensitive imaging techniques to image the urinary tract.

Cystourethroscopy (CUSC) is another commonly used imaging modality to diagnose and follow-up on the urinary bladder wall and the urethra. This technique needs to be performed with local anaesthesia. A cystoscope (similar to endoscope) is slowly inserted through the urethra as the camera gathers images of the bladder and the urethra. It is commonly used for imaging bladder lesions, cystitis, bladder cancer etc. CUSC has limited imaging depth and cannot provide 3-dimensional structural information of bladder wall [120]. Also, the image resolution is not the best.

Ultrasound (US) imaging is used for bladder imaging very frequently. US imaging allow higher imaging depths compared to CUSC, and are used to image the lower urinary system in women to detect genuine stress urinary incontinence. Additionally, US imaging also gives information of bladder wall lesion and bladder cancer [121]. The US imaging proved to be an efficient tool for Burch colposuspension [122-124]. 2D and 3D imaging of urinary bladder was compared. The mean error in measurement of urinary bladder dimensions in 3D imaging is 4.9% while the mean error was 27.5% in 2D ultrasound imaging. First febrile urinary tract infection in young children is detected using US imaging [125, 126].

Optical imaging such as multiphoton microscopy, fluorescence was used to study urinary bladder [127-129]. Computational model of urinary bladder was developed to study the propagation of ultraviolet and blue wavelength [130]. This model was used for differentiation of healthy and diseased urinary bladder using fluorescence imaging. Absorption and scattering properties of porcine urinary bladder was studied from 400 nm to 1800 nm. Optical coherence tomography was used to differentiate inflammation, hyperplasia and neoplasia [131].

The different imaging techniques currently used for bladder imaging have several limitations including use of ionizing radiation, invasive imaging, shallow imaging depth, poor resolution, inadequate sensitivity etc. Therefore, a non-invasive, safe and better imaging alternative is required. PAI combines the rich optical contrast with high ultrasound resolution [31, 33, 132-134]. Commercial clinical ultrasound systems with PA imaging capabilities are developing rapidly. PAI is being explored as an alternative for bladder imaging due to its non-invasive and non-ionizing nature [135]. PAI uses intrinsic contrast within the body (blood, melanin, water etc.) or uses extrinsic contrast agents like organic dyes, nanoparticles, etc. [136-142]. For bladder imaging there is no intrinsic contrast provided from the urine, therefore extrinsic contrast agents are needed. Dyes like methylene blue and indocyanine green (ICG) have been used for bladder imaging. For non-invasive monitoring of vesicoureteral reflux, ICG enhanced carbon nanotubes are injected into the rats and monitored over time [143]. PAI of canine urinary bladder was studied to determine the presence of muscularis propria invasion. Murine urinary bladders were imaged for oxygen saturation computation [144]. PA imaging was combined with fluorescent imaging for quantification of ICG accumulation in urinary bladder. Vasculature of urinary bladder is imaged using photoacoustic microscopy [145, 146]. However, all these are shown with laboratory

prototype photoacoustic imaging systems, with limited clinical translational capability. Clinical ultrasound systems with combined light delivery and image acquisition are being explored for various biological applications recently [37, 147-149].

In this study, photoacoustic imaging of urinary bladder was combined with ultrasound imaging for obtaining structural and functional information. A clinical dual mode ultrasound and photoacoustic imaging system was used. For deep tissue imaging, 1064 nm light was used as the photoacoustic excitation source. Contrast agent (black ink) was injected into the urinary bladder to show the structural PA imaging. Sagittal and transverse sections of urinary bladder were shown in PA and ultrasound modes up to a depth of 2 cm. The feasibility of using combined US and PA imaging of a bladder was demonstrated in rats and the feasibility of the same for VUR was demonstrated in phantom imaging as modelling this disease in live rat is challenging. Along with structural information PA imaging can also provide functional information, which was demonstrated with combined US-PA imaging of nanoparticle clearance from the body. Nanoparticle clearance from the body was studied by injecting commercially available gold nanorods, through tail vein injection and tracking the accumulation of nanorods in the bladder at regular intervals over a time period of 150 minutes.

4.2.2 Materials and Methods

4.2.2.1 US-PA imaging system

The imaging system used for combined US-PA imaging is as described in section 3.2.4. The schematic of the imaging system is shown in Fig. 21.

Figure 21: Schematic representation of the combined ultrasound-photoacoustic imaging system for *in vivo* imaging.

4.2.2.2 Animal preparation

Healthy adult male Sprague Dawley rats of weight 225 ± 25 g (aged 8-10 weeks) were obtained from InVivos Pte. Ltd., Singapore. The animal preparation is similar to section 4.1.2.3. Hair from the abdominal area of the rat was removed using commercial depilatory cream before imaging. A 23G urinary bladder catheter was inserted through the urethra and was secured with tissue glue to prevent leakage. During experiments, animal was maintained under anaesthesia with 0.75% of isoflurane gas (Medical Plus Pte Ltd, Singapore) along with oxygen (1.2 L/min). The mixture was delivered through a nose cone wrapped with a breathing mask covering the mouth and nose of the animal completely. The animal was placed in supine position and images (US + PA) were recorded with the handheld probe. After image acquisition, the animal was euthanized with an overdose of pentobarbital.

4.2.2.3 Structural imaging of bladder

A rectangular chicken tissue slice (7 X 6 cm) of approximately 1 cm in thickness was placed on the abdominal region of the rat. Black ink was used as the PA contrast agent and saline was used as control. Combined US and PA image of the bladder was taken before injection of dye or saline in both the transverse and the sagittal plane. 0.8 mL of black ink or saline was injected into the bladder through the catheter. After 10 minutes, the bladder was imaged in both the planes. Once, the PA signal from the bladder was clearly visible, depth imaging was performed. For depth imaging chicken tissue slices of 0.5 cm thickness was stacked one by one until a depth of 2 cm. Combined US and PA images were obtained and stored in both the transverse and sagittal plane.

4.2.2.4 Flow imaging

Vesicoureteral reflux is a condition where there is a reverse flow of urine from the bladder to the kidney via the ureters. To demonstrate the feasibility of imaging the flow of urine from the bladder into the ureters, phantom imaging was done as modelling the disorder in rats is very difficult. A phantom was made with LDPE tubes of diameter 1.6 mm and latex material in ellipsoidal shape to mimic the urinary system. The tubes mimic the urethra and the ureters; the latex material was made in the ellipsoidal shape to mimic the urinary bladder. The phantom was placed on a chicken tissue slice of thickness 3 cm and a 0.5 cm chicken tissue slice was placed on top. Combined US and PA imaging was done. The handheld probe was moved along the tissue slice to identify the ureter mimicking phantom with US imaging. The handheld probe was held in the same position. The bladder mimicking phantom was filled with black ink slowly through the urethral phantom. Slowly the black ink flows through the ureter phantom. A video showing the combined US and PA imaging flow of the black ink from the bladder through the ureters was recorded from the screen.

4.2.2.5 Clearance imaging

Combined US and PA imaging can be used for imaging nanoparticle clearance from the body. To demonstrate this 0.2 mL of gold nanorods (35 μ M/mL) (Sigma Aldrich, USA) of average size 10 nm was injected into the bloodstream of the rat through a tail vein injection. The gold nanorods have high optical absorption at 1064 nm therefore strong PA signal is expected. The bladder catheter was closed to prevent leakage through the urethra. A 1 cm thick chicken tissue was placed on the abdominal region for optimising light delivery. The bladder was imaged to obtain combined US and PA images in the sagittal and the transverse plane over a period of 150 minutes at regular intervals of 15 minutes. The Signal to noise ratio (SNR) was calculated from the saved PA images and plotted across time. For comparison an ellipsoidal phantom of 1.5 mL volume (roughly the size of a full bladder) containing 0.2 mL of gold nanorods was imaged and the SNR was calculated. For phantom imaging the phantom was placed on top of a 3 cm thick chicken tissue slice and covered with a 1 cm thick chicken tissue slice to mimic the *in vivo* imaging condition. The SNR value from the phantom imaging can be compared with the SNR calculated from the *in vivo* bladder data to determine the rate of clearance of gold nanorods from circulation.

4.2.2.6 Laser safety

The coupling efficiency of the fiber bundle at 1064 nm was ~50%. The energy of light falling on the surface was ~45 mJ per pulse. The total area of illumination was ~5 cm² [109]. Therefore the fluence can be calculated approximately as 9 mJ/cm² on the surface (skin). The maximum permissible energy on skin at 1064 nm is 100 mJ/cm² according to the American National Standards Institute [76]. Therefore, all studies were done well within the prescribed safety limit.

4.2.3. Results and discussions

Non-invasive imaging of urinary bladder (UB) is very useful for different applications. Here, we show the structural imaging of the UB with combined US and PA imaging. Additionally PA imaging of back flow of the urine from the bladder to the kidney is shown with a bladder and ureter mimicking phantom for VUR imaging. Then, non-invasive imaging of clearance of nanoparticles (which has photoacoustic contrast) from the body through bladder is shown. For experiments, the rat bladder was imaged along the sagittal and transverse planes as shown in Fig. 21.

Black ink was used as a PA contrast agent due to its strong absorbance at the near infrared wavelength window. For experiments, 1064 nm was chosen as the imaging wavelength. Due to weak absorption of light and lower scattering, the penetration depth of light is high at this wavelength. For urinary bladder imaging a higher imaging depth is needed especially in human, as the abdominal muscle is of few centimetres thickness. Saline was used as control as it does not have any photoacoustic contrast at 1064 nm. Fig. 22(a) and 22(b) show the combined US-PA image of the urinary bladder (before injection of saline) of the rat in the sagittal and transverse plane, respectively. Fig. 22(c) and 22(d) show the US-PA image of UB after injection of saline in the sagittal and transverse planes, respectively. It is to be noted from the images that there is no PA signal from the bladder both before and after injection of saline. However, from the ultrasound image the structure of the bladder can be clearly delineated and it can be noted that there is an increase in the size of the bladder after injection of saline through the catheter. Figs. 22(e) and 22(f) show the sagittal and transverse combined US-PA images before injection of black ink. It can be observed that the bladder structure can be seen in US, but there is no PA signal from the bladder. Figs. 22(g) and 22(h) show the combined US-PA images of the UB after injection of black ink through the catheter

in the sagittal and transverse plane, respectively. After injection, there was a strong PA signal from the bladder wall from the black ink. The grayscale image represents the US image and the colour is a representative of PA signal. Structural information will help in identifying bladder wall deformity, which can indicate bladder disorders.

Figure 22: Structural imaging: Sagittal plane imaging - (a,b) Combined US-PA image of the rat urinary bladder before and after injection of saline; (e,g) combined US-PA image of the rat bladder before and after injection of black ink. Transverse plane imaging – (b,d) Combined US-PA image of the rat urinary bladder before and after injection of saline; (f,h) combined US-PA image of the rat bladder before and after injection of black ink.

In rats the bladder is located very close to the skin. In humans, the bladder is located much deeper depending on the muscular and fat content of the person, typically 2-3 cm. Therefore, combined US-PA imaging of bladder at different depths (using chicken tissue) is shown in both sagittal and transverse planes. Fig. 23(a-d) show combined US-PA images of the bladder at different depths of 0.5 cm, 1 cm, 1.5 cm, and 2 cm in the sagittal plane. Figs. 23(e-h) show the combined US-PA images of the rat urinary bladder in the transverse place at different depths (0.5 cm, 1 cm, 1.5 cm, and 2 cm). From the images it can be observed that the PA signal is very prominent when the imaging depth is 0.5 cm and 1 cm. After 1 cm, the PA signal decreases with increase in

depth. However, there is sufficient PA signal for imaging from the bladder even at higher depths. It is also important to note the fluence of imaging is much lesser than MPE advised by ANSI for 1064 nm. Therefore, the imaging depth can be improved further by increasing the laser power, thereby increasing the fluence or by using extrinsic contrast agent with higher light absorption at 1064 nm.

From the stored beam-formed images, SNR of the PA signal from the bladder was calculated at different depths. SNR is given by $SNR = \frac{V}{n}$, where V is the PA signal amplitude and n is the standard deviation of the background noise. 30 frames were averaged and the SNR was calculated from the averaged image. The calculated SNR was plotted against depth of imaging as shown in Fig. 23(i). It can be noted from the plot that the maximum SNR is at 1 cm depth, which is expected, as the illumination angle of 15° works best at 1 cm [109, 149]. Even at 2 cm imaging depth the SNR value is approximately 40, which is sufficiently high for imaging. As stated earlier, since imaging was done at a very low energy, there is scope for getting higher SNR values at higher energy levels.

Vesicoureteral reflux is a very common urinary tract disease. Currently, this is imaged with the help of X-Ray cystography, during micturition and normal state. However, during X-ray imaging, the patient gets exposed to ionizing radiation. Therefore, PA imaging is a non-invasive and a safer imaging alternative for imaging and diagnosing vesicoureteral reflux. Fig. 24(a-b) show the ureter phantom images before and after the back flow from the urinary bladder. In rats the ureter diameter is very small, so phantom of 1 mm diameter was used for experiments. But in humans, the ureter can be easily located with ultrasound and vesicoureteral reflux can be imaged with PA imaging with a suitable PA contrast agent under different conditions to determine when it occurs.

Figure 23: Depth imaging: (a-d) Combined US-PA images (sagittal plane) of the rat bladder at depths of 0.5 cm, 1 cm, 1.5 cm, and 2 cm, respectively. (e-h) Combined US-PA images (transverse plane) of the rat bladder at depths of 0.5 cm, 1 cm, 1.5 cm, and 2 cm, respectively. (i) Plot of SNR vs imaging depth.

Another application for which PA imaging of bladder can be used is for monitoring clearance of nanoparticles from the body. There are not many non-invasive techniques to monitor clearance of contrast agent from the body. PA imaging can be used for clearance study if the nanoparticle provides a good photoacoustic signal (high optical absorption) at the specific wavelength. For this experiment commercially available gold nanorods were introduced into blood through a tail vein injection and the bladder of the rat was continuously monitored for 150 min at 15 minute intervals in both sagittal and transverse plane. The SNR at different time points was calculated and plotted vs time in Fig. 25(i). Figs. 25(a-d) and Figs. 25(e-h) show the combined US-PA image of the UB at different time intervals of 0 min, 15 min, 60 min, and 120 min in the sagittal and transverse planes, respectively. From Fig. 25(i) it can be observed that the SNR keeps gradually increasing with time. Also from the images the PA signal increase can be

observed. With increase in time the nanoparticles tends to settle down gradually and PA signal can be observed from the bottom part of the bladder. A phantom with similar volume of a full bladder (1.5 mL) with 0.2 mL of gold nanorods was made and the SNR was calculated. The phantom imaging was mimicking the *in vivo* imaging scenario very closely. The SNR value was found to be 122. The SNR calculated at the end of five hours was found to be 56. Therefore, it can be roughly estimated that the average clearance time for ~50% gold nanorods from circulation is around 150 min. The rate of elimination depends on the enhanced permeation and retention properties of the nanoparticle. For different particles the clearance rate will vary.

Figure 24: Vesicoureteral imaging: (a and b) Combined US-PA image of before and after the reverse flow of the black ink in the ureter mimicking phantom.

In this part we have discussed about the different prospects of using clinical US-PA imaging system for urinary bladder imaging. PA imaging can be used for structural and functional imaging of the bladder. Imaging of vesicoureteral reflux (common bladder disorder) using PAI is focused. However, with the right contrast agents (targeted nanoparticle for bladder cancer); we believe PAI can be used extensively for bladder imaging and can aid in the diagnosis of bladder related disorders. However, there are some limitations with the current format of clinical PA imaging. One of the limitations is the fiber based light delivery; it limits the maximum light energy that can be transported to the transducer end. Therefore, this limits us from operating the system at

the highest possible energy within the ANSI permissible limit. With higher laser energy better images can be obtained and also depth of imaging can also be improved. Hence, cost-effective fibres withstanding high energy is much needed for improved imaging. Another problem related to the optical fiber is that its flexibility determines the angle of light illumination, which affects the optimum imaging depth greatly. Therefore, it is important to have a very flexible fiber which allows different light illumination angles and use the optimum light illumination angle for the required depth of imaging. Another limitation for the current imaging system is its inability to provide good 3-D imaging. It is very difficult to track the hand motion, therefore, the 3D imaging can be done with a raster scan by motorised stage. A better 3D imaging platform may provide better information regarding the structure and function of the bladder.

Figure 25: Nanoparticle clearance imaging: (a-d) Combined US-PA images of the bladder at 0 min, 15 min, 60 min, and 120 min, respectively, in the sagittal plane after tail vein injection of gold nanorods; (e-h) Combined US-PA images of the bladder at 0 min, 15 min, 60 min, and 120 min, respectively, in the transverse plane after tail vein injection of gold nanorods. (i) Plot showing SNR vs time of imaging (in minutes) after tail vein injection of gold nanorods.

4.2.4. Conclusion

Combined ultrasound photoacoustic clinical imaging systems are being explored for different biological applications. Integration of handheld photoacoustic imaging together with ultrasound system currently used in clinic will support bedside monitoring. Even though PA imaging of urinary bladder has been discussed earlier, its feasibility through clinical ultrasound system is shown here. Structural and functional urinary bladder imaging in small animals is demonstrated with the handheld combined ultrasound and photoacoustic imaging system. Up to 2 cm imaging depth is shown. Also, diagnosis of vesicoureteral reflux, a common bladder disorder, is demonstrated with phantom imaging. Additionally, functional PA imaging of clearance of nanoparticles from circulation is also demonstrated by monitoring the accumulation of gold nanorods in the bladder.

Chapter 5

Contrast agents for PA imaging⁵

5.1 Dual modal ultrasound/photoacoustic contrast agent

5.1.1 Introduction

PA imaging has been integrated with other imaging modalities, such as ultrasound, magnetic resonance imaging, fluorescence imaging etc. In particular, PA imaging systems can be easily integrated with US imaging systems, as they both rely on ultrasound transducers for data acquisition and image formation. Till now, most of the PA imaging systems used are either custom made research systems which doesn't give access to raw channel data [63] or clinically incompatible system which uses single element UST for data acquisition [150]. Recently, commercial clinical ultrasound imaging systems are available which allows dual modal US-PA imaging [37, 147, 149]. So, there is an increasing need for dual mode contrast agents in US-PA imaging [151-153]. Dual-modal US/PA imaging systems have found applications in identifying tumors in lungs [154]. US imaging was used for *in vivo* identification and monitoring of orthotopic lung tumors whereas PA imaging was used to characterize oxygenation in the tissues surrounding the tumor. These agents have also been used for imaging sentinel lymph nodes [39, 63] in patients with breast cancer. US enables visualization of lymph node size and shape while PA helps to determine whether a lymph node is a sentinel lymph node by imaging the accumulated dye. Other examples include imaging

⁵ Part of the work presented in this chapter has been submitted to *Scientific Reports*. D. Das, **K. Sivasubramanian**, C. Yang, and M. Pramanik, "On-chip generation of microbubbles in photoacoustic contrast agents for dual modal ultrasound/photoacoustic in-vivo animal imaging", Major Revision, *Scientific Reports* (2018).

of thyroid glands [155], joints [156] and angiogenesis [157]. Instead of dual-modal US/PA imaging, co-administration of both microbubbles and PA contrast agent dyes can be done but if the microbubbles are injected after the PA dye injection, the water phase of the microbubbles will eject the PA dye from the region of interest. Alternatively, we may wait for some time for injecting the microbubbles after the PA dye or vice versa. However, this may delay the patient's diagnosis and there is no set time limit for such a procedure. Another substitute to co-administration is pre-mixing the microbubbles with the drug payload such as dyes, nanoparticles, etc in the syringe itself just prior to injection. However, we cannot ensure how uniformly the drug payload will disperse in the water phase of the microbubbles. Moreover, the size distribution of the microbubbles may change from the original size distribution in the mixing process. Commercial ultrasound contrast agent such as SonoVue is sold in a sealed syringe by the manufacturer. For clinical purposes, the ingredients in the SonoVue solvent cannot be pre-mixed with FDA approved dyes such as methylene blue prior to injection. Thus, US/PA dual-modal contrasts, providing combinatorial diagnosis, using commercially available ultrasound platforms have become increasingly important [151-153].

Microbubbles with a stabilized shell comprising of surfactants [158] or phospholipids [159, 160] are used as US contrast agents [20] for diagnostic applications, such as imaging the liver and heart [161]. These microbubbles may have different gas cores like nitrogen, perfluorobutane, etc [19]. For US/PA based dual modal imaging, microbubbles have been integrated with several exogenous contrast agents. For instance, microbubbles have been shelled with porphyrin [162] and gold nanoparticles [163], synthesized in methylene blue [152] solution as well as encapsulated with black ink [164].

Traditionally, microbubbles are produced using bulk mixing methods like mechanical agitation or sonication [165]. However, the bubbles produced through these methods are polydisperse. A narrow size distribution is desirable because the resonance frequency of the microbubbles depends on its diameter [166]. We looked into the generation of microbubbles using microfluidics in methylene blue [152] and black ink [167] solutions which have been used as photoacoustic contrast agents. A flow-focusing junction [160, 168, 169] is used for the generation of microbubbles. There are several advantages of using microfluidics for generating microbubbles. Firstly, the diameter, production rate, and the number of microbubbles to be delivered can be accurately controlled, which can be used to regulate how much microbubbles are to be released *in vivo* for targeted drug delivery [170]. Secondly, microfluidic devices can be miniaturized for placement within a catheter so that the microbubbles can be directly injected into the therapeutic region of interest [165, 169]. Thirdly, the gas shell composition of the microbubbles as well as the external liquid phase composition can be precisely customized. Microfluidics-produced microbubbles have been used for sonothrombolysis [171], mouse tail injection [172], etc. Microfluidic devices have also been used for the generation of nanobubbles [168] for high resolution ultrasound imaging in the mouse aorta. We envisage the use of microfluidic devices for the continuous generation of nanoparticle coated monodisperse-microbubbles [173, 174] as dual modal US/PA contrast agent.

We report the generation of nitrogen microbubbles using a flow-focusing junction-based microfluidic device in photoacoustic contrast agents, namely, methylene blue and black ink. Stability analysis of the microbubbles in both solutions was first done using optical microscope. Thereafter, more microbubbles were generated and withdrawn into glass syringes which were injected into low density polyethylene (LDPE) tubes for

phantom imaging. Finally, *in vivo* animal imaging was performed by injecting the microbubbles into the urinary bladder of rats.

5.1.2 Methods

5.1.2.1 Microbubbles generation in methylene blue and black ink

High purity nitrogen gas (Leeden National Oxygen Ltd., Singapore) was used as a dispersed phase for the generation of the microbubbles. The microbubbles were generated in two different photoacoustic contrast agents which were used as the continuous phases. The first continuous phase contained MB hydrate (Sigma, Singapore) at a concentration of 10 gm/L in deionized (DI) water. First, a DI water mixture is prepared which contains 5% PEG-40 (Sigma, Singapore), 10% glycerol (Sigma, Singapore), 10% propylene glycol (Sigma, Singapore) and 1% polyvinyl alcohol (PVA) (Sigma, Singapore). Thereafter, 10 gm of MB hydrate is added per litre of DI water. For the BI solution, first BI was diluted 50 times in DI water. Thereafter, 5% PEG-40, 1% glycerol and 1% propylene glycol were added to the BI and DI water mixture. With 10 percent of propylene glycol and glycerol concentration in BI solution, debris was observed in the microfluidic channels. Only when the 10 percent concentration was reduced to 1 percent, the debris were no longer visible. The BI was purchased from a stationary shop in Nanyang Technological University, Singapore. In the absence of PVA, the microbubbles in the MB solution adhered to the walls of the glass syringe which would coalesce to form bigger bubbles over time and were getting destroyed post-syringe injection. We observed adding PVA to the BI solution was not necessary as the microbubbles did not adhere to the walls of the glass syringe, were still in the microscale and gave a good US signal. Flow rates of 2 ml/hr and 8 ml/hr were provided for the MB and BI solutions, respectively by a syringe pump (Longer) to the two inlets of the polydimethylsiloxane (PDMS)-based microfluidic device at the flow-

focusing junction. Although the standard commercial vial of SonoVue contains 5 ml of solvents, the required dose varies depending on the part of the body which is to be examined. For instance, for the B-mode imaging of cardiac chambers in adults, the recommended dose is 2 ml. So, to produce microbubbles in 2 ml quantity of MB and BI, it will take 1 hr and 0.25 hrs, respectively. The PDMS-based microfluidic device was fabricated using standard soft-photolithography. The width of the flow-focusing junction was $10\ \mu\text{m}$ and widths of all the inlets and outlets were $300\ \mu\text{m}$. The top view schematic of the microchannels is shown in Fig. 1(a). The height of the microchannels was $25\ \mu\text{m}$. For both MB and BI solutions, a pressure of 1 bar was delivered to the gas inlet of the microfluidic device from a nitrogen cylinder by using a pressure regulator (Spectron, Singapore). An inverted microscope (Nikon Eclipse TE2000) was used for observing the microbubbles generation at the flow focusing junction of the microfluidic device. The inverted microscope is fitted with a high-speed CMOS camera (Phantom Miro 310) which could record images at frames up to 650,000 frames per second (fps).

Figure 26: (a) Dimensions of the flow-focusing junction. (b) Schematic of the experimental set-up. (c) Microbubbles collected from the outlet of the microfluidic device. (d) and (g) show the monodisperse microbubbles generated in methylene blue (MB) and black ink (BI), respectively. (e), (f) shows the microbubbles in MB solution at time, $t = 0\text{ min}$ and $t = 30\text{ min}$. (h), (i) shows the microbubbles in BI solution at time, $t = 0\text{ min}$ and $t = 30\text{ min}$.

5.1.2.2 Dual modal US-PA imaging system

The dual modal imaging system is described in detail section 3.2.4. The schematic of the imaging set up is shown in Fig. 27 (a). The imaging system is similar to the one described in section 3.2.4.

Figure 27: (a) Schematic representation of the photoacoustic imaging system with clinical ultrasound platform. OF - optical fiber bundle; USM - clinical ultrasound machine; UST - ultrasound transducer. The optical fiber holder houses the 2 output optical fiber bundle for illumination of tissue and ultrasound transducer for signal acquisition. An anaesthesia machine supplying isoflurane and oxygen was used to maintain the animal under anaesthesia for the duration of the experiments. (b) Photograph of rat showing, sagittal and transverse imaging planes.

5.1.2.3 Phantom imaging

To test the efficiency of the microbubbles in methylene blue and black ink as combined US and PA contrast agents, phantom imaging was done. The laser was tuned to 675 nm for microbubbles in MB solution, as MB has maximum absorption around this

wavelength [109], and the laser was tuned to 1064 nm for imaging microbubbles in BI solution for demonstrating deep tissue imaging although BI has high absorption at most wavelengths in the visible and near infrared regions. Imaging at 1064 nm will be beneficial for deep tissue imaging as the penetration depth of light is high at this wavelength. Both the solutions containing microbubbles were injected in to LDPE tube of 1.68 mm inner diameter and the tube was placed in a water tank. Light illumination was done in free space and the area of illumination was approximately 1 cm in diameter. The transducer surface was placed in parallel to the tube and perpendicular to the light illumination. US image was captured first, and then combined US and PA image was captured. The setup is shown in Fig. 29(a). For control saline, commercial ultrasound contrast agent, methylene blue solution and black ink solution was used. US only and combined US-PA images were captured for the same. For all phantom experiments, images were saved as beam formed data type and the imaging depth was set to 2 cm. 50 image frames were saved for each of the sample.

5.1.2.4 Animal preparation and imaging

To prove the efficiency of the microbubbles in methylene blue and black ink as dual modal contrast agents *in vivo* imaging of rat urinary bladder was carried out. Healthy adult female Sprague Dawley rats of weight 300 ± 50 g (aged 12-14 weeks) were obtained from InVivos Pte. Ltd., Singapore. The animal preparation is described in section 4.1.2.3. Then, the abdominal area is cleared of hair using commercial hair removal cream. Subsequently, a 23G urinary bladder catheter was inserted through the urethra and was secured using tissue glue to avoid leakage. The animal was maintained under anaesthesia throughout the experiments.

The animal is placed facing upwards and a 0.5 cm thick chicken tissue slice is placed on the shaved region and the probe is placed on the animal and both US and combined

US+PA images are obtained before injection in the transverse and sagittal planes for control images [shown in Fig. 21(b)]. 0.8 mL volume of microbubbles in MB and BI solutions are injected through the catheter. US, and combined US+PA images of the bladder were obtained in the transverse and sagittal planes. Saline was used as control. All the images were saved as beam-formed datatype. Total imaging depth was set as 3 cm. 50 imaging frames were saved for each experiment.

5.1.2.5 Laser safety

The optical fiber bundle has a coupling efficiency of ~50% at both 675 nm and 1064 nm laser wavelengths. At 675 nm the energy of light falling on the surface is 20 mJ per pulse with an illumination area of ~3 cm² and 1064 nm the energy is 45 mJ per pulse with an illumination area of ~5 cm². According to the American National Standards Institute (ANSI) the maximum permissible energy on skin at 675 nm and 1064 nm are 20 mJ/cm² and 100 mJ/cm², respectively[76]. The fluence calculated for the experiments are 6 mJ/cm² and 9 mJ/cm² at 675 nm and 1064 nm, respectively. Therefore, we are well within the safety limit.

5.1.3 Results and Discussion

The top view of the microfluidic flow focusing junction, which was used for the microbubble generation, is shown in Fig. 26(a). A schematic of the experimental set up is shown in Fig. 26(b). This set up is described in detail in the Methods (1) section. At the flow focusing junction of the microfluidic device, microbubbles were generated in methylene blue [Fig. 26(a)] and black ink [Fig. 26(f)] solutions at flow rates of 2 ml/hr and 8 ml/hr, respectively. The difference in flow rates is due to the fact that with MB; 10% glycerol and 10% propylene glycol, was used compared to BI which contained 1% glycerol and 1% propylene glycol. A higher percentage of glycerol and propylene glycol is desirable as it retains monodispersity of the microbubbles[160] but with BI,

debris and pigments were observed at higher percentages at the flow-focusing junction of the microfluidic device under the microscope. As a result of this, the microbubbles production rate was not stable and it eventually led to choking of the microchannels.

Figure 28: Size distributions of the microbubbles in methylene blue (MB) at (a) time, $t = 0$ min and (b) time, $t = 30$ min. Microbubbles in black ink (BI) at (c) time, $t = 0$ mins and (d) time, $t = 30$ mins.

At 25°C, the viscosity of glycerol (947 centipoise) and polypropylene glycol (49.1 centipoise) is higher than the viscosity of water (1 centipoise). Hence, the higher percentage of glycerol and propylene glycol in the MB made it more viscous than the BI which are used as the continuous phases in our experiments. As a result, a lower flow rate had to be used for the MB compared to BI. Higher flow rates resulted in the leakage of MB from the microfluidic device. For BI, it was observed that at lower flow rates, the gas phase does not pinch off and hence, no microbubbles were formed. The production rates were in the order of 10^4 microbubbles/sec and 10^6 microbubbles/sec in

the MB and BI, respectively. Also, the microbubbles population count was of the order of 10^8 microbubbles/ml and 10^5 microbubbles/ml for the MB and BI solutions, respectively. These were quantified by observing the flow-focusing junction using the high speed camera at 60,000 frames per second at 256 x 128 resolution as shown in Figs. 26(d,g). The counting was done just downstream of the flow-focusing junction. There is a slowdown of microbubble's velocity downstream because the width of the microchannel expands to 300 microns as shown in Fig. 26(a).

The microbubbles were collected from the outlet as shown in Fig. 26 (c) using glass syringes (Fortuna Optima, Singapore) and then pushed out between two cover slips for observation under the microscope. Initially, monodisperse nitrogen microbubbles of diameter 6 to 7 μm [Figs. 26(d,g)] were produced at the flow focusing junction for the both continuous phases. However, when the same batch of microbubbles was collected from the outlet and observed under the microscope, they were found to be polydispersed. A plot of the size distribution of the microbubbles is shown in Fig. 28 at time, $t = 0$ min [Figs. 28(a,c)] and $t = 30$ min [Figs. 28(b,d)] after the formation of the microbubbles. In the MB solution, 67% of the microbubbles had a diameter in the size range of 3 to 6 μm . After 30 minutes, it was observed that 70% were in the 3.5 to 5.5 μm range. So, the size of the microbubbles roughly remains the same. However, there is a 30% drop in the total number of microbubbles. As for the BI solution, 80% were in the size range of 6 to 12 μm . After 30 minutes, 85% were in the size range of 4.5 to 20 μm . Also, the number of microbubbles reduced by around 50%. The polydispersity and loss in the total number of microbubbles occurred due to the coalescence and fragmentation of the same, which is regarded as Ostwald ripening[176] effect. This effect can be prevented by using a mixture of glycerol and propylene glycol[160]. It has been reported that the monodispersity can be maintained by increasing the inter-

bubble spacing[176]. However, in our experiments, the microbubbles were withdrawn into glass syringes and pushed out between cover slips. So, the spacing between the microbubbles could not be controlled and consequently, the monodispersity was compromised.

Figure 29: (a) Schematic representation of the low-density polyethylene (LDPE) tube with sample immersed in a water bath. The red circle on the tube denotes the area of laser illumination. (b)-(g) US images of saline, SonoVue, MB, MB+μ-bubbles, BI, BI+μ-bubbles, respectively, and (h)-(m) Combined US+PA images of saline, SonoVue, MB, MB+μ-bubbles, BI, BI+μ-bubbles, respectively.

Microbubbles in MB and BI, were collected from the outlet of the microfluidic device and injected into LDPE tubes for phantom imaging. The US+PA signals obtained were also compared with different samples. Figure 29 shows the US and the combined US+PA images of the different samples obtained from the photoacoustic imaging system with clinical ultrasound platform. The different samples that were imaged includes saline as general control; SonoVue, a commercial ultrasound contrast agent as ultrasound control, plain MB and BI as control for PA imaging and finally, the test samples of MB+microbubbles and BI+microbubbles. Figures 29(b-g) show the US

image of the tube containing saline, sonovue, MB, MB+microbubbles, BI, and BI+microbubbles, respectively. Figures 23(h-m) show the combined US and PA images of the tube phantom containing saline, SonoVue, MB, MB+microbubbles, BI, and BI+microbubbles, respectively. The gray scale image represents the ultrasound image and the coloured region represents the PA image.

From saline (control) sample there is no noticeable US and PA signal as expected [Figs. 29(b,h)]. Only the ultrasound signal from the outline of the tube is visible as shown in Fig. 29 (b). There is no US and PA signal from within the tube. From the commercial ultrasound contrast agent, SonoVue, very strong ultrasound signal can be noticed but there is no evident PA signal as shown from Figs. 29(c,i). MB, a well-established PA contrast agent gave no US signal but strong PA signal (laser excitation at 675 nm wavelength) as evident from Figs. 29(d,j). On the other hand, MB solution with microbubbles gave strong US and PA signal as seen in Figs. 29(e,k). BI sample gave no US signal but very strong PA signal at 1064 nm as shown in Figs. 29(f,i). However, one of the dual modal contrast agent BI with microbubbles gave very strong US and PA signal at 1064 nm as depicted in Figs. 29(g,m). It is to be noted that the ultrasound signal from the LDPE tube, as shown in Fig. 29(b), is visible in all the images and should not be confused with the US signal generated from the microbubbles. The US signals from the microbubbles are highlighted in Figs. 29(e,g). Additionally, it is to be noted that the bubbles have a tendency to float up and accumulate towards the top surface of the tube. When the concentration of microbubbles is high, there is a very strong ultrasound signal from the microbubbles. Simultaneously, a weaker PA signal is observed from underneath the microbubbles in the tube. This is observed in Figs. 29(k,m). However, this effect is less pronounced in Fig. 29(m) because the total number of microbubbles was lesser in BI than in MB as stated earlier. Additionally, the PA

signal is observed only in a small area because the laser illumination area (~1 cm) is small compared to the imaging area of the transducer (3.85 cm).

Figure 30: *In vivo* rat urinary bladder images (US+PA and US). Sagittal plane: (a-c) before and after injection of saline, (d-f) before and after injection of MB+μ-bubbles, (g-i) before and after injection of BI+μ-bubbles. Transverse plane: (j-l) before and after injection of saline, (m-o) before and after injection of MB+μ-bubbles and before and after injection of BI+μ-bubbles.

The signal to noise ratio (SNR) for the various phantoms were calculated and shown in Table 3. SNR is given in decibels (dB) by $SNR = 20 \times \log_{10}(V/n)$, where V represents the PA signal amplitude, n stand for the standard deviation of the background noise. The SNR is calculated after averaging 30 image frames. The saline solution had the least SNR values for both US and PA imaging, 28 dB and 14 dB, respectively. SonoVue had the maximum SNR for ultrasound signal (71 dB), but negligible SNR for PA imaging (24 dB). On the other hand the SNR for US signal was 30% less for MB+microbubbles (50 dB) and 14% less for BI+microbubbles (62 dB) in comparison to SonoVue. The SNR for PA signal of the dual mode contrast agents was compared with the respective dyes and there was no significant change in the SNR for PA signal due to the presence of the microbubbles. The reduction in the PA SNR values was 1.5% due to the presence of the microbubbles in MB (61 dB) and remained the same in BI

(61 dB). There is no significant change in SNR because the microbubbles are not distributed uniformly throughout the tube. There are pockets in the tube in which the dyes are concentrated near the top surface of the tube. SNR for PA signal is calculated from the entire tube, which makes the SNR of PA signal from the MB+microbubble and BI+microbubble similar to that of pure MB and BI. However, if SNR is calculated from the PA signal right below the region where the bubbles are located there will be a noticeable difference.

Table 4: Comparison of signal to noise ratios (SNR) of US and PA signals for the various samples in tube phantom.

Sample	US (dB)	PA (dB)
Saline	28	14
SonoVue	71	24
Methylene blue (MB)	29	62
Methylene blue (MB) + microbubbles	50	61
Black ink (BI)	24	61
Black ink (BI) + microbubbles	61	61

For *in vivo* experiments, rat urinary bladder imaging was performed to demonstrate the use of dual mode contrast agents. The rat bladder was imaged in the transverse and the sagittal plane. Fig. 30 shows the bladder imaging in both the planes before and after injection of saline, MB+microbubbles, BI+microbubbles. Before injection, combined (US+PA) images were obtained. After injection, US only and combined (US+PA) images were obtained. It can be noted from Figs. 30(a-c) and 30(j-l) that saline does not provide any US and PA signal although there is significant increase in the size of the bladder after injection images. Figures 30(d-f) and 30(m-o) show the before and after

injection images of MB+microbubbles in sagittal and transverse planes, respectively. From the after injection images, it can be observed that there is a strong US signal from microbubbles and a strong PA signal from MB. The bubbles float and move towards the top wall of the bladder. As a result, the US signal from the microbubbles is strong from the top part of the bladder. The PA signal appears from the bottom part of the bladder wall as the dye stains the bladder walls. Therefore PA signal appears from the bottom part. Similar results were obtained with BI+microbubbles as observed from Figs. 30(g-i) and 30(p-r) showing the before and after injection images of the BI+microbubbles in sagittal and transverse planes. The SNR was calculated and shown in Table 4. For saline, the SNR of US and PA signal was observed to be negligible. On the other hand, the US and PA imaging SNR values for MB+microbubbles were 48 dB and 33 dB, respectively, and the SNR for US and PA imaging with BI+microbubbles were 51 dB and 43 dB, respectively. There was a significant difference between the SNR recorded from phantoms and *in vivo*. There was a 4% and 46% difference between the SNR for ultrasound and PA for MB. Similarly, there was a 17% and 29% difference between the SNR for ultrasound and PA for BI. This occurs because there is lot of scattering and diffusion in the tissue.

Table 5: Values of signal to noise ratios (SNR) of US and PA signals from the *in vivo* animal images (average of both sagittal and transverse planes).

Sample	US (dB)	PA (dB)
Saline	26	22
Methylene blue (MB) + microbubbles	48	33
Black ink (BI) + microbubbles	51	43

With increase in concentration (microbubbles/ml) and microbubble sizes (μm), the mean intensity of the ultrasound images increases [177]. As it can be seen from Fig. 22, the total count of microbubbles in MB is much higher than that of BI. However, the microbubbles are also smaller in diameter in MB compared to BI. Hence, the US imaging SNR values of the *in vivo* images of the urinary bladder stays relatively the same for both MB and BI solutions in the presence of microbubbles. The US images of the urinary bladder are shown in Figs. 24(e,h) and 24(n,q). However, for the tube phantom data as shown in Table 1; it was observed that US imaging SNR values of the BI+microbubbles is much higher than MB+microbubbles. This may be attributed to the complex wall interactions between LDPE tubes which has a hydrophobic surface [178] and microbubbles. A comparative analysis of the effect of MB and BI [179] on microbubbles within LDPE tubes would also be needed which is beyond the scope of this work. In the context of the PA signal, Mansik *et al.* [152] reported that the PA signal decreases with the presence of microbubbles. This agreed well with the PA imaging SNR values obtained from our phantom imaging experiments. However, PA imaging SNR value did not drop significantly as can be seen from Table 1 when comparing MB and MB+microbubbles as well as BI and BI+microbubbles. This was observed as microbubbles coalesced to get bigger which occasionally burst while being pushed along the LDPE tubes. Moreover, the bubbles did not distribute evenly throughout the illuminated area of the tube.

5.1.4 Conclusion

We demonstrated that US/PA dual modal contrast agents can be synthesized using flow-focusing junction based microfluidic devices. Nitrogen microbubbles of diameters 6 to 7 μm were generated in two photoacoustic contrast agents: MB and BI. The monodispersity as well as the microbubble concentration (number of microbubbles per

ml) in photoacoustic contrast agents could be precisely maintained. First, optical microscope was used to examine the size distributions of the microbubbles in both contrast agents. Next, the microbubbles in both solutions were injected into LDPE tubes for phantom imaging experiments. Finally, *in vivo* animal imaging of rat's urinary bladder was performed. US signals were seen from the top of the urinary bladder as the microbubbles tend to float upwards. PA signals were seen from the bottom part of the urinary bladder. The effect of microbubbles, on both US and PA signals, was also examined.

5.2. Near Infrared light-responsive liposomal contrast agent⁶

5.2.1 Introduction

The PA image contrast is based on the optical absorption by endogenous molecules (hemoglobin, melanin, etc.), however, these molecules show relatively weaker light absorption in the near-infrared region. Nevertheless, image contrast can be improved with the aid of exogenous contrast agents having strong optical absorption in the NIR wavelength region. Several metallic, inorganic, organic nanoparticles, quantum dots, carbon nanotubes etc. were demonstrated for deep tissue photoacoustic imaging [141, 180-182]. Contrast agents not only helps in enhancing the photoacoustic signals, but it can also be useful for targeted molecular imaging, drug delivery, therapy etc [183, 184]. Research work is in progress to find a stable, non-toxic, biocompatible, and yet powerful exogenous contrast agent for photoacoustic imaging.

Among various nanocarriers, liposomes exhibit stable encapsulation of therapeutics, long circulation half-time[185, 186] and these agents are clinically successful [187, 188]. The most conventional composition of liposomes contains dipalmitoylphosphatidyl choline (DPPC) as its main constituent. DPPC with a phase transition (transition from gel to liquid-crystalline state) temperature at around 41°C, enables release of the encapsulated therapeutic from liposome in response to temperature [189]. However, one of the major constraint of these liposomes is their inability to initiate the drug release at the target site due to inadequate temperature control *in vivo* [190]. One of the various attempts to modulate the temperature at

⁶ Part of the work presented in this chapter has been published in *Journal of Biomedical Optics*. **K. Sivasubramanian**, M. Mathiyazhakan, C. Wiraja, P. K. Upputuri, C. Xu, and M. Pramanik, "Near Infrared light-responsive liposomal contrast agent for photoacoustic imaging and drug release application," *Journal of Biomedical Optics* 22(4), 041007 (2017).

physiological conditions has resulted in stimuli responsive liposomes that can be externally manipulated by light. Simplicity, clinical adaptability, and unparalleled spatio-temporal control on the drug release being the key aspects, gold nanostructures are coupled with liposomes to make them better responsive to light [191, 192]. Earlier a photoresponsive system by encapsulating gold nanoparticles (AuNPs) and the model drug Calcein within temperature sensitive liposomes by a simple thin film hydration method was developed. However, this method suffers from the low encapsulation of nanoparticles and the drug and is also limited by penetration depth of visible light for clinical applications [193].

In this part, we describe the synthesis, and characterization of temperature sensitive liposome coated with gold nanostars (AuNSs) with a strong absorption peak at 690 nm. The strong absorption in the NIR window makes it suitable for photoacoustic imaging, making it ideal for dual functional imaging agent as well as drug delivery carrier for therapeutics. Coating the liposomes surface with AuNSs, facilitates optimal encapsulation of the therapeutics within the liposomes and the optical properties of AuNSs at NIR wavelength facilitates deep tissue drug delivery [191, 194]. In addition, the proximity of the AuNSs to the lipid bilayer is more effective even in low intensities of light, to cause the disruption of liposomes and subsequent drug release. Despite the advantages, till date, limited studies have been reported on gold coated liposomes. Troutman et al and Leung et al., reported the synthesis of gold coated liposomes using ascorbic acid as the reducing agent. Although they were successful in demonstrating wavelength selective drug release, the efficacy of the system was not tested on *in vitro* or *in vivo* systems [194]. Gold coated liposomes reported by Rengan et al. demonstrate light triggered drug release by a photothermal effect, requiring a prolonged exposure of laser [195]. Along with drug release applications, liposome-gold hybrid systems are

being actively investigated for their applications in photothermal therapy and multimodal imaging [196, 197]. However, for photoacoustic imaging gold coated liposomes has not been reported. We have demonstrated AuNS coated liposomes as a potential exogenous contrast agent for deep-tissue PAT imaging. The near instantaneous controlled drug delivery from AuNSs coated liposomes using photoacoustic effect is also explored.

5.2.2 Materials and methods

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-sn-glycero-3 phosphocholine (MPPC) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabama, US). HAuCl₄ and Paclitaxel were purchased from ACROS Organics. Fresh mouse blood was collected from animal facility center, Nanyang Technological University. All other chemicals unless otherwise mentioned were purchased from Sigma-Aldrich. Deionized (DI) water was purified by a Millipore Milli-DI water purification system.

5.2.2.1 Synthesis of Liposomes coated with gold nanostars

Temperature sensitive liposomes were first prepared by the conventional thin film hydration method as reported previously [195]. Briefly 7.95, 0.65 and 1.39 mg/mL of DPPC, MPPC, and DSPE-PEG2000, respectively were dissolved in chloroform (in a molar ratio of 86:10:4). The organic phase was evaporated at 37°C under reduced pressure in a rotary evaporator (Buchi, Switzerland) to form the lipid bilayer. For Paclitaxel loaded liposomes (PTX-L), 1 mg of PTX was dissolved in the organic phase. For Calcein loaded liposomes (Cal-L), the thin lipid film was hydrated with 1 mL calcein solution (60 mM, 7.4 pH). The plain liposomes (PL) were hydrated with PBS. The liposomes were then subjected to size extrusion using 200 nm polycarbonate

membrane filters (Avanti Polar Lipids). The unencapsulated Calcein solution was removed by gel filtration chromatography with PD-10 desalting columns (GE Healthcare). The liposomes were then centrifuged at 12000 rpm at 4°C for 60 minutes to form a concentrated pellet.

To coat the liposomes with AuNSs, liposomal pellet with a total lipid concentration of 2 mg was reconstituted in 1 mL of 1 M HEPES buffer (pH 7.4). 50 μ L of HAuCl₄ was added to the solution and was left at room temperature in dark under constant shaking at 400 rpm. After 1 hour, the liposomal solutions coated with AuNSs were centrifuged at 12000 rpm 4°C for 30 minutes to remove the free AuNSs and excess HEPES buffer. Plain liposomes coated with AuNSs (AuL), calcein loaded liposomes coated with AuNSs (Cal-AuL), PTX loaded liposomes coated with AuNSs (PTX-AuL) were thus formed by the above procedure. The liposomes were stored at 4°C and were used within 48 hours.

5.2.2.2 Characterization of liposomes loaded with AuNSs

The hydrodynamic diameter, polydispersity, and zeta potential of liposomes was measured using dynamic light scattering technique (DLS, ZetaPALS analyser, BIC). Average of 6 measurements was reported for each sample. The concentration of gold in liposomes was quantified by Inductively Coupled Plasma Spectrometer (ICP, Prodigy ICP Spectrometer). 100 μ L of liposome was digested overnight in a mixture of HCl and HNO₃ (in a ratio 3:1). The sample was then diluted to 10 mL and was quantified against the gold standards. The morphology of the liposomes and the presence of AuNSs coated on the lipid bilayer surface were determined by transmission electron microscopy (TEM). 10 μ L of sample solution was placed on 200 mesh formvar carbon coated copper grids (FCF-200-Cu, Electron Microscopy Sciences) and was air-dried. After negative staining with 3% uranyl acetate for 1.5 minutes, the grids were

washed with DI water. Finally, the grid samples were air-dried overnight and were imaged using FEI Tecnai G2 Spirit Bio-TWIN electron microscope, operated at 120 kV. The morphology of the AuNSs was confirmed using high resolution TEM (HRTEM) imaging. For this, the liposomes were lysed with Triton X 100 and AuNSs were collected after centrifuging the samples at 15,000 g for 15 minutes at 4°C. The AuNSs were then re-dissolved in DI water and 5 µL of the AuNSs solution was placed on 200 mesh formvar carbon coated copper grids (FCF-200-Cu, Electron Microscopy Sciences). The grids were completely dried before imaging with TEM (FEI G2 spirit, 120 kV, America). Phospholipid assay was performed to determine the total lipid concentration by mass. Loading efficiency was estimated from the on the standard curves constructed for known concentration of phospholipids, calcein and paclitaxel, at an excitation max of 535 nm, 480 nm, and 230 nm, respectively. The loading efficiency was determined by

$$LE = \frac{Wt\ of\ calcein\ encapsulated}{Wt\ of\ final\ total\ lipids} * 100$$

5.2.2.3 Cell culture

B16-F10 mouse melanoma cells (ATCC) were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose 4.5 g/L and 4.0 mM L-Glutamine) supplemented with 10% FBS and penicillin/streptomycin (100 U/ml) at 37°C with 5% CO₂.

5.2.2.4 Photoacoustic Spectrum of liposomes loaded with AuNSs

An optical parametric oscillator, OPO (Continuum, Surelite), pumped by a Q-switched 532 nm Nd:YAG laser was used as an excitation source for photoacoustic spectrum measurements. It generates 5 ns duration pulses at 10 Hz repetition rate with wavelengths tunable from 670 nm to 2500 nm. The solution containing liposomes

coated with AuNSs (AuL) was placed inside a low-density polyethylene tube with an inner diameter (ID) of 0.59 mm and outer diameter (OD) of 0.78 mm. The sample containing tube and the single-element ultrasound transducer (UST) (V323-SU/2.25 MHz, 13 mm active area, and 70% nominal bandwidth, Panametrics, Olympus) were immersed in water for coupling of PA signals to UST. The tube was irradiated with wavelengths ranging from 670 - 900 nm with a 10 nm step size. Respective PA signals were collected using the UST and these signals were subsequently amplified, and band pass filtered by an Amplifier/Filter unit (A/F) (Olympus-NDT, 5072PR). Finally, the output from A/F unit was digitized with a data acquisition card (GaGe, compuscope 4227) and stored in the computer. Peak-to-peak PA signal was then normalized with the laser energy for each wavelength and was plotted against the wavelength to generate the PA spectrum.

5.2.2.5 Photoacoustic signal from liposome and animal blood

The PA spectrum of liposomes coated with AuNSs (AuL) shows peak at 690 nm. So, for further PA experiments, the laser was tuned to 690 nm and the incident laser energy density was maintained at 5 mJ/cm². To measure PA signal, the AuNSs coated liposome sample/animal blood was placed inside a 0.59 mm diameter LDPE tube. The sample containing tube was irradiated with 690 nm laser beam. Respective PA signals from liposome and blood samples were collected using the 2.25 MHz UST.

5.2.2.6 Deep-tissue photoacoustic imaging experiment

The LDPE tubes (~1 cm long) filled with fresh mouse blood and AuNSs coated liposomes (2 mg/mL) were embedded in a chicken breast tissue. A photoacoustic tomography (PAT) imaging system was used for tissue imaging experiments [71]. The tissue surface was irradiated with an expanded 690 nm laser beam having energy density 5 mJ/cm² (< ANSI safety limit 20 mJ/cm²) and the PA signals were collected

by continuously rotating the 2.25 MHz UST around the sample in a circular geometry. The scan time was 1 minute. PA signals were collected when the tubes were placed at a depth of 1 and 2 cm beneath the laser-illuminated chicken tissue surface. The cross-sectional PAT images were reconstructed using a delay-and-sum algorithm [198].

5.2.2.7 Photoacoustic imaging of melanoma cells

Photoacoustic imaging of the B16F10 melanoma cells was done using a clinical ultrasound-photoacoustic imaging system. The cells were labelled with AuNSs coated liposomes with a total lipid concentration of 100 $\mu\text{g/mL}$ for 48 hrs and unlabelled cells were used as control. The labelled and the unlabelled cells were then trypsinized and centrifuged at 400 g for 5 mins to obtain the cell pellet. The experimental set up uses a clinical research ultrasound system (ECUBE 12R, Alpinion, South Korea) with photoacoustic imaging capability. In order to operate the system in the photoacoustic mode, laser excitation needs to be provided as well as a trigger which synchronizes the excitation of the laser and the signal acquisition by the ultrasound transducer.³⁰ With a final concentration of 1×10^5 cells/mL in 1 mL transparent tubes at an imaging depth of 2 cm, the cells were irradiated at 690 nm. Real time images were acquired at a frame rate of 5 frames per second (fps). The PA signals were acquired using a linear array transducer (L3-12 transducer), consisting of 128 array elements with centre frequency of ~ 8.5 MHz.

5.2.2.8 Drug Release Studies

1 mL Calcein encapsulated AuNSs coated liposome solution (Cal-AuL) was taken in a quartz cuvette and was irradiated for varying time points (0-60 seconds) with the OPO laser with an energy density of 10 mJ/cm^2 . 10 μL liposomal solution was collected after every 5 seconds and was replaced with fresh aliquots. Collected samples were transferred to an Eppendorf tube containing distilled water and were placed onto an ice

box to avoid further release. The samples were centrifuged at 15000 rpm to remove AuNSs coated liposomes and the supernatant was transferred to 96 well plates. The percentage of Calcein released was quantified by measuring the fluorescence at an excitation and emission wavelengths of 480 nm and 520 nm using a fluorescence spectrophotometer (H4 Synergy). The experiment was repeated with Calcein encapsulated liposome without the AuNSs (Cal-L). Both Cal-L and Cal-AuL were treated at 37°C and the amount of Calcein released was measured as a control. In the same way, PTX release assay was also performed using PTX encapsulated AuNSs coated liposome solution (PTX-AuL) and PTX encapsulated liposome without the AuNSs (PTX-L). The percentage of PTX released was quantified by measuring the absorbance at 230 nm using a UV spectrophotometer (Spectramax). Both PTX-L and PTX-AuL were treated at 37°C and the amount of PTX released was measured as a control.

5.2.2.9 Qualitative Analysis of *in vitro* Calcein Release

For *in vitro* experiments, B16F10 mouse melanoma cells were incubated with Cal-AuL (with a final concentration of 40 µg total lipids/mL) and were incubated for 6 hours at 37°C with 5% CO₂. The cells were washed trice with PBS and then irradiated with the OPO laser at 690 nm for 30 seconds per group with an energy density of 10 mJ/cm². Plain liposomes, free calcein and Cal-L treated groups were used as controls. To verify the Calcein release from the liposomes, the cells were imaged using LX71 inverted fluorescence microscope (Olympus) and Retiga-2000R CCD camera, one hour post laser treatment. It is to be noted that the settings (500 ms exposure time and 3x gain) for the all green fluorescent images, at 20x image magnification was kept constant throughout the imaging. Finally, the images were processed using the ImageJ software to normalize background signal. The calcein release intensity was quantified using the

fluorescence microplate reader with an excitation and emission wavelengths of 480 nm and 515 nm, respectively.

5.2.2.10 Cytotoxicity Assay

The B16F10 mouse melanoma cells were incubated with Paclitaxel loaded liposomes coated with AuNSs (with a final concentration of 40 μg total lipids/mL) and were incubated for 6 hours at 37°C with 5% CO₂. The cells were washed trice with PBS and then irradiated with an OPO laser at 690 nm for 30 seconds per group with an energy density of 15 mJ/cm². Plain liposomes, free PTX and PTX-L treated groups were used as controls. To test the effectiveness of the release of Paclitaxel, cell viability assay was performed using Alamar Blue, 24 hours post laser treatment.

5.2.3. Results and Discussion

Temperature sensitive liposomes were prepared using the conventional thin film hydration method and coated with AuNSs by reducing the gold precursor, HAuCl₄ using HEPES buffer. HEPES was found to reduce the Au³⁺ ions in HAuCl₄ to Au⁰ thus forming AuNSs [199] and this zerovalent state leads to the deposition of AuNSs on the surface of the liposomes [200]. The liposome was characterized as shown in Fig. 31. The presence of AuNSs on the surface of the liposomes imparts characteristic optical properties to the AuNSs coated liposomes making it responsive to light. The maximum absorbance of light occurred at 690 nm measured from the UV-Vis spectroscopy as shown in Fig. 31(a). The plain liposomes and AuNSs coated liposomes were characterized for their size, surface potential and morphology. The hydrodynamic diameter measured by the dynamic light scattering method was found to be 141.4 ± 0.5 and 113.2 ± 0.7 nm as shown in Fig. 31(b), while the surface charge was -28.38 ± 1.25 and -22.37 ± 0.23 mV for plain liposomes and AuNSs coated liposomes, respectively. The TEM images indicated the presence of AuNSs on the surface of the liposomes and

the size of the AuNSs liposomes from the TEM images was found to be 96.77 nm [Fig. 31(c)], which is comparable to the hydrodynamic diameter measured. The HR-TEM images clearly revealed the branched star shaped morphology of the AuNSs as shown in Fig. 31(d). From the ICP results, the concentration of gold in AuNSs coated liposomes was found to be 160 μ M. The loading efficiency of calcein and PTX was found to be 24% and 25%, respectively.

Figure 31: Characterization profile of AuNSs coated liposomes represented by the (a) UV-Vis absorption spectrum of AuNSs coated liposomes, (b) Hydrodynamic diameter of AuNSs coated liposomes and Plain liposomes, (c) TEM image of AuNSs coated liposomes, and (d) HR-TEM image of AuNSs. Scale bar: 50 nm.

The photoacoustic spectrum revealed that there is a strong photoacoustic signal at 690 nm as shown in Fig. 32(a), indicating high optical absorbance at this wavelength. Based on this observation, the laser excitation was fixed at 690 nm for the further experiments. At 690 nm, it was found that the photoacoustic signal from 2 mg/mL AuNSs coated liposomes was 2 times stronger than that from of the animal blood as shown in Fig. 32(b). The peak to peak photoacoustic signal from blood and AuNSs coated liposomes

were found to be 0.21 V and 0.45 V, respectively. The potential use of AuNSs coated liposomes as PA contrast agent and their effective imaging depth at a wavelength of 690 nm, was tested. As shown in Fig. 32(c), two LDPE tube phantoms were filled with fresh mouse blood and AuNSs coated liposomes (2 mg/mL), respectively. They were sandwiched between chicken breast tissue of varying depths (1 and 2 cms). Figs. 32(d,e) shows the cross-sectional photoacoustic tomography images of the tubes. The SNR values of blood and AuNSs coated liposomes at 1 cm depth was 13 and 25, respectively as shown in Fig. 32(d). Similarly, SNR values of blood and AuNSs coated liposomes at 2 cm depth was 6 and 14, respectively as shown in Fig. 32(e). Our results indicate that AuNSs coated liposomes are promising contrast agents for PAT, providing good PA signal enhancement ($\sim$ two times) and image contrast in biological tissues even at 2 cm depth.

Figure 32: (a) PA spectrum of AuNSs coated liposomes (AuL), (b) PA signals collected from blood and AuNSs coated liposomes, (c) Photograph of the LDPE tubes filled with 2 mg/mL AuNSs coated liposomes and animal blood placed inside (sandwiched) chicken breast tissue, (d-e) Cross-sectional PAT images of the tubes at (d) 1 cm, (e) 2 cm imaging depth.

The tube containing the B16F10 melanoma cells in the media is shown in Fig. 33(a). The combined ultrasound and B-scan PA image (beam-formed image) of the tubes containing the unlabelled and the labelled melanoma cells are shown in Figs. 33(b) and (c), respectively. The greyscale image represents the ultrasound image and the colours represent the photoacoustic image. Melanin has an intrinsic photoacoustic signals. It has a good contrast against blood in a wide range of wavelength (600-800 nm). The bright red spots on the image show the melanoma cells. It can be seen that in both the labelled and the unlabelled samples, the cells can be imaged. The difference in the signal intensity might not be so obvious from the PA/US image, because the system normalises the images by default. If the PA images alone are extracted from it, PA signal would be more pronounced and obvious. This can be noted from Fig 33 (d,e) which shows the normalised PA images of both the labelled and the unlabelled melanoma cells. Also, for clarity, the SNR from both the images is reported in Fig. 33(f). The SNR was defined as the amplitude of the PA signal from the melanoma cells divided by the standard deviation of the background noise, $SNR = V/n$, here V is the PA signal amplitude, and n is the standard deviation of the background noise. As shown in Fig. 33(f), the signal-to-noise ratio (SNR) of the unlabelled B16F10 mouse melanoma cells is 45, while that of the AuNSs coated liposomes labelled B16F10 mouse melanoma cells is 174. This 3.8 time better signal intensity of the labelled cells further confirms that AuNSs coated liposomes can be used as a photoacoustic contrast agent for cell labelling and tracking.

Next, the drug release capability of the liposomes was tested. Cal-AuL and PTX-AuL solutions were exposed to the NIR pulsed laser for varying time points (0-60 seconds) with an energy density of 10 mJ/cm². The percentage of drug release from liposomes and AuNS coated liposomes is shown in Figs. 34(a) and (b), respectively. From the

figures, it can be observed that, the presence of AuNS enabled $72.09 \pm 0.88\%$ and $76.08 \pm 3.28\%$ release of encapsulated Calcein and PTX, respectively within 60 seconds under the laser exposure. While the liposomes without AuNSs had only $\sim 10\%$ drug release under the same experimental condition.

Figure 33: (a) Photograph of tube containing B16F10 mouse melanoma cells in media, (b) Combined ultrasound and B-scan PA image unlabelled B16F10 mouse melanoma cells, (c) combined ultrasound and B-scan PA image of AuNSs coated liposomes labelled B16F10 mouse melanoma cells, (d) Normalised B-scan PA image of unlabelled B16F10 mouse melanoma cells, (e) Normalised B-scan PA image of AuNSs coated liposomes labelled B16F10 mouse melanoma cells, and (f) Graph comparing the SNR values of unlabelled B16F10 mouse melanoma cells (UNL) and AuNSs coated liposomes labelled B16F10 mouse melanoma cells (L).

Figure 34: Percentage of Drug Release (a) Release of Calcein from Cal-AuL and Cal-L. (b) Release of PTX from PTX-AuL and PTX-L upon laser irradiation at 690 nm with an energy density of 10 mJ/cm^2 at a pulse repetition rate of 10 Hz.

Fluorescence imaging using B16F10 melanoma cells were done to demonstrate the release of calcein from the liposomes triggered by light induced pressure waves (photoacoustic waves). B16F10 melanoma cells were incubated with free calcein solution (120 μM), liposomes containing calcein, and AuNSs coated liposomes containing calcein overnight. After washing the cells with PBS to remove the particles that were not taken up by the cells, each of the treated groups were exposed to laser at 690 nm at 10 mJ/cm^2 for 30 seconds. When subject to a laser source at 690 nm, the absorbed light causes a temperature rise, which generates pressure waves (photoacoustic waves). These pressure waves' leads to the disruption of liposomes, releasing the encapsulated drug.³³ Laser untreated groups were compared against the laser treated groups. This is shown in Fig. 35. It was observed that free calcein stained the cells in both laser treated and untreated groups, no fluorescence was observed in both groups in case of cells incubated with liposomes containing calcein. However, the cells incubated with AuNSs coated liposomes containing calcein, significant increase in fluorescence was observed in laser treated groups while no fluorescence was observed in groups unexposed to laser. As reported earlier, the local temperature increase in the nanoparticle microenvironment generates microbubble cavitation that disrupts the liposomal membrane to release the encapsulated drug [191, 193]. Likewise, the presence of AuNSs in the liposomes results in Calcein release due to microbubble cavitation. It is also to be noted that the rise in temperature of the bulk solution containing the same concentration of AuNSs coated liposomes was found to be less than 4°C upon laser exposure of about 10 mins. This further validates that the encapsulated drug is released by the liposome disruption due to the pressure wave generation and eliminates the possibility of drug release due to rise in temperature of

the bulk liposomal solution. At a concentration of 60 mM, calcein self-quenches within the liposomes and the cells are non-fluorescent. As the laser triggers the AuNSs coated liposomes containing calcein, the released calcein stains the cells and can be detected as its fluorescence recovers.

Figure 35: *In vitro* Release of Calcein from Cal-AuL into the cytoplasm of B16F10 melanoma cells with and without laser exposure. The cells were irradiated with a laser for 30 seconds at an energy density of 10 mJ/cm². Figures 5(a-d) represent the DAPI stained cell nuclei of control group without any laser exposure; Figures 5(e-h) represent the Calcein stained cell cytoplasm of control group without any laser exposure; Figures 5(i-l) represent the DAPI stained cell nuclei of laser treated group; Figures 5(m-p) represent the Calcein stained cell cytoplasm of laser treated group; Figure 5p represents the cells stained with Calcein released from Cal-AuL after laser exposure. Blue: Nuclei, Green: Cell Cytoplasm. Scale bar: 100 μ m.

To test the biocompatibility of the AuNSs coated liposomes, B16F10 mouse melanoma cells were incubated with AuNSs, free drug paclitaxel, liposomes loaded with paclitaxel, AuNSs coated liposomes loaded with paclitaxel for 24 hrs. The cells were then washed with PBS and the cell viability was assessed using Alamar blue assay

against the untreated cells. Similarly, cells treated with laser at 690 nm for 30 sec were tested for viability 24 hours post the laser treatment to see the efficacy of the drug release. These results are depicted in Fig. 36. It was found that the cells that were not exposed to laser did not show any significant changes in the viability. While the cells treated with PTX showed significant decrease in cell viability in both control and laser treated groups, about 20% cell death was observed from cells treated with AuNSs coated liposomes without PTX. This clearly indicates that the microbubble cavitation generated due to the presence of AuNSs in the liposomes has led to the release of PTX into the cell cytoplasm, which in turn have resulted in the cell death. However, the highest percentage of cell death was observed from cells treated with AuNSs coated liposomes containing paclitaxel upon laser exposure. Cells treated with liposomes with paclitaxel did not show significant changes in the cell viability ensure stable encapsulation of PTX within liposomes. This shows that the AuNSs causes significant drug release from the liposomes.

Photoacoustic imaging can be used for theranostic applications when used with a suitable contrast agent. Here, we explore the theranostic potential of near-infrared light-sensitive liposomes coated with gold nanostars (AuNSs) for both imaging and drug release applications using photoacoustic imaging system. The liposome being amphiphilic can encapsulate both hydrophobic and hydrophilic molecules. Irradiation by laser at a particular wavelength, triggers the rapid release of the contents encapsulated in the liposomes due to local heating and pressure wave formation (photoacoustic wave) by the AuNSs. The pressure waves can also be used for PA imaging as well. However, further *in vivo* studies in small animal needs to be done to prove the efficacy of this system for clinical applications.

Figure 36: Cell viability study of B16F10 melanoma cells treated with PTX Au-Liposomes with and without laser exposure. The sample was irradiated with laser for 30 seconds at 15 mJ/cm² energy density. (PL - Plain liposomes, AuL - AuNS coated liposomes, PTX - free drug paclitaxel, PTX L - liposomes loaded with paclitaxel, PTX-L AuL – AuNS coated liposomes loaded with paclitaxel).

5.2.4 Conclusion

In this part, we have demonstrated that AuNSs coated liposomes show strong absorption in the NIR region and can be used as contrast agent for photoacoustic imaging of deep tissues. The drug release study promises that it can be used to deliver both hydrophilic and hydrophobic drugs effectively. Thus we present AuNSs coated liposomes as a dual modal therapeutic and contrast agent responsive to NIR light. Further use of these particles for *in vivo* small animal models is under investigation for its potential use in the clinics.

6. Conclusion and future directions

6.1 Conclusion

In this dissertation the possibility of a clinical dual modal ultrasound and photoacoustic imaging system by combining it with two different lasers (PLD and OPO) for different applications including sentinel lymph node and urinary bladder is presented. Dual modal (US+PA) contrast agents were developed with microbubbles in organic dyes and gold nanostars coated liposomes was developed and studied as a potential photoacoustic contrast agent.

The PLD was successfully integrated with the clinical ultrasound system to facilitate high framerate PA imaging at 7000 fps at 803 nm wavelength. The high framerate was shown with the flow imaging of black ink in a tube phantom. The same was tested with OPO laser, where only 10 fps (max) PA imaging is possible. Theoretically, with a frame rate of 7000 fps flow rate up to 134.4 m/s can be captured. It was demonstrated that with 7000 fps frame rate, the flow can be visualized and accurate flow measurements can be done from the beam-formed PA images obtained at various flow rates 3 cm/s to 14 cm/s (controlled by syringe pump), which cannot be observed with the OPO-PAT system.

The OPO was combined with the clinical ultrasound imaging system for dual mode handheld US-PA imaging. Light delivery from the laser was coupled to the transducer with a bifurcated optical fiber. The optimum light delivery angle, distance, etc. were optimized with Monte Carlo simulations for different depths of imaging as these factors have a significant effect on imaging. Different simulation scenarios were considered for the different parameters affecting the imaging. Experimental validation was

provided for the different illumination angles at various SLN depths for a given fiber to tissue and fiber to probe distances. From simulations, it was shown that different illumination angles are required to obtain maximum absorbance in SLN phantom. Based on that, several 3D printed probe holders were designed and PA imaging was done using the clinical ultrasound platform. From experiments it was confirmed that the angle of light delivery plays an important role with respect to the depth of imaging at a given fiber to tissue distance.

The commercial clinical ultrasound system was successfully used for combined US and PA pre-clinical imaging of SLN and urinary bladder in small animals (rat). For SLN imaging, MB and ICG were used as contrast agents as they have high optical absorption in the NIR wavelength region. Additionally, MB is FDA approved for clinical SLNB making it easy to translate to clinic. To confirm that the SLN images were because of the dyes that fills the lymph nodes PA spectroscopy was done. SLN imaging at varying depths of up to 1.5 cm was performed. Additionally, non-invasive needle guidance for FNAB was performed by tracking the needle in real time with PA imaging. This will be beneficial to eliminate SLNB surgery and further avoiding the complications related to it.

Structural and functional urinary bladder imaging in small animals was demonstrated with black ink as the contrast agent. Bladder imaging of up to 2 cm imaging depth was also shown. Additionally, diagnosis of vesicoureteral reflux, a common bladder disorder, was demonstrated with phantom imaging. Furthermore, functional PA imaging of clearance of nanoparticles from circulation was demonstrated by monitoring the accumulation of gold nanorods in the bladder.

Contrast agents are very essential for PA imaging. First contrast agent that was explored was a dual modal ultrasound-photoacoustic agent. The feasibility of synthesizing

US/PA dual modal contrast agents using flow-focusing junction based microfluidic devices was shown. Monodisperse nitrogen microbubbles of diameters 6 to 7 μm were generated in two well established photoacoustic contrast agents (MB and BI). First, the size distribution of the microbubbles was examined under an optical microscope. MB+ μ -bubbles and BI+ μ -bubbles were injected for into LDPE tube phantoms for PA imaging. Lastly, *in vivo* imaging of rat's urinary bladder was performed. The effect of microbubbles, on both US and PA signals, was also examined.

The second contrast agent that was tested was AuNSs coated liposomes. It has strong optical absorption in the NIR region and is a potential contrast agent for photoacoustic imaging of deep tissues. The drug release study indicates that it can be used to deliver both hydrophilic and hydrophobic drugs effectively. Thus, AuNSs coated liposomes as a dual modal therapeutic and contrast agent responsive to NIR light was demonstrated.

6.2 Future directions

There are many more hurdles to overcome before having PA imaging in clinic, like portable systems, efficient light delivery, more contrast agents, etc. There are also multiple untapped applications where PA imaging can be used. The long term goal is to make PA imaging a non-invasive bed-side imaging technology which can be made possible by portable lasers and ultrasound systems. In the future, we propose to use portable OPO lasers and high energy PLD lasers for better imaging. Recently, tunable OPO lasers are available in the market (*e.g.*, portable OPO laser by Opotek). Using such portable lasers, we propose to use highly flexible optical fiber which allows light delivery at greater angles of up to 60° at least. This will enable PA imaging at various depths.

The limited number of FDA approved contrast agents is a limiting factor in translating PA to the clinic. In future, we propose to use contrast from naturally available food products like fruit juices, food dyes, coffee etc. These type of contrast agents can be used for label free imaging of the gastrointestinal tract especially without FDA approval. We aim to explore and identify the peak optical absorption of the various dyes in the visible and the NIR region and further test its potential as a photoacoustic contrast agent for clinical use. Methods to make these dyes target specific will aid in other applications such as tumor imaging etc.

We also propose to use the high frame rate imaging system for imaging rapidly moving objects. Metastasis of melanoma cells will be indicated by the circulating tumor cells of the original tumor. The melanoma CTCs has intrinsic high optical absorption in the NIR range compared to blood. High framerate imaging can detect really fast moving the CTCs in the blood flow which can be easily missed by the current PA imaging systems due to their slow imaging rate (200 fps). Also, the high framerate imaging can be used for imaging blood clots in blood to diagnose deep vein thrombosis. The blood clot has at least two times more PA signal than the blood in the NIR range (803 nm) which can help to identify and differentiate the blood clot and aid in non-invasive label free imaging.

Appendix: Modification in the Monte Carlo

Simulation

Launching of photons in conventional MCML and MCEO is a pencil beam. In the experimental setup, a fiber bundle was used. Hence, the simulations here also needed the fiber bundle. The fiber bundle tips were generated as follows. The fiber core diameter was $185\text{ }\mu\text{m}$ (with cladding the diameter was $200\text{ }\mu\text{m}$). The fiber bundle fabricated for experiments had 800 tips randomly positioned in a rectangular region of $4\text{ cm} \times 0.142\text{ cm}$. The core of $185\text{ }\mu\text{m}$ was approximated to a point source. Hence, for the launch of photons in simulation, 800 points were generated with a minimum distance of $200\text{ }\mu\text{m}$ between each of them. A random vector of dimension 1×2 was initialized. The values of the random vector range from 0 to 1. Vector was scaled to 4 and 0.142 to match the probe dimension. A new random vector was generated and scaled. The distance between the previously generated fiber tip and the new vector was computed. If the distance between the vectors was greater than $200\text{ }\mu\text{m}$, then the new vector was appended to set final fiber tip positions. The list was grown until 800 points were obtained. Two sets of fiber tips were generated, which formed a look-up table. An equal number of photons was launched from each of the fiber tips.

Once launched the position of the photons (x and y) was initialized with the value from the look-up table and z was set to 0. Direction cosines of the photon (u_x , u_y , and u_z) were computer based on the launch angle. With the help of a random number, the further spin angle due to the NA was computed for each photon. Spinning of the photon was done in the local co-ordinate system and then the direction cosines were converted back to the global co-ordinate system. Co-ordinate system conversion and spin done here are explained in the original MCML simulation. The photon's conventional steps

of scattering and absorption are illustrated in Fig. 6. Exclusively for study 4, when the photons hopped beyond the tissue size ($-5 \geq x \leq 5$ and $-3.5 \geq y \leq 3.5$) they were terminated. When the photon was to be transmitted from layer 2 (which is tissue), 36% of it was reflected back into the medium. The reflection of the photon back into the medium was by changing the sign of the direction cosine of the z-axis (u_z).

Figure 37: Flow chart of Monte Carlo with embedded sphere (MCES) with modifications highlighted. s is the step-size, ξ is the random number, d_{ob} is the distance to the object (sphere), d_b is the distance to boundary, and μ_t is transmission coefficient.

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Peer reviewed journals

1. D. Das, **K. Sivasubramanian**, C. Yang, and M. Pramanik, "On-chip generation of microbubbles in photoacoustic contrast agents for dual modal ultrasound/photoacoustic in-vivo animal imaging", Major Revision, *Scientific reports* 2018.
2. **K. Sivasubramanian**, V. Periyasamy, R.A. Dienzo, and M. Pramanik, "Hand-held, clinical dual mode ultrasound - photoacoustic imaging of rat urinary bladder and its applications", *Journal of Biophotonics* e201700317, (2018).
3. **K. Sivasubramanian**, V. Periyasamy, and M. Pramanik, "Non-invasive sentinel lymph node mapping and needle guidance using clinical handheld photoacoustic imaging system in small animal," *Journal of Biophotonics* 11(1), e201700061 (2018) .
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6. **K. Sivasubramanian**, M. Mathiyazhakan, C. Wiraja, P. K. Upputuri, C. Xu, and M. Pramanik, "Near Infrared light-responsive liposomal contrast agent for photoacoustic imaging and drug release application," *Journal of Biomedical Optics* 22(4), 041007 (2017).
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1. **K. Sivasubramanian**, V. Periyasamy, D. R. Austria, and M. Pramanik, "Photoacoustic cystography using handheld dual modal clinical ultrasound photoacoustic imaging system," *Proc. SPIE* 10494, 1049442 (2018).
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3. **K. Sivasubramanian**, V. Periyasamy, K. K. Wen, and M. Pramanik, "Optimising probe holder design for sentinel lymph node imaging using clinical photoacoustic system with Monte Carlo simulation," *Proc. SPIE* 10064, 100645N (2017).
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