Investigations into hyperspectral and hybrid-optical imaging for bio-applications

Lim, Hoong Ta

2017

Lim, H. T. (2017). Investigations into hyperspectral and hybrid-optical imaging for bio-applications. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/70073>

<https://doi.org/10.32657/10356/70073>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**INVESTIGATIONS INTO HYPERSPECTRAL AND
HYBRID-OPTICAL IMAGING FOR BIO-APPLICATIONS**

LIM HOONG TA
SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

2017

INVESTIGATIONS INTO HYPERSPECTRAL AND HYBRID-OPTICAL IMAGING FOR BIO-APPLICATIONS

LIM HOONG TA

School of Mechanical and Aerospace Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

Acknowledgements

I would like to take this opportunity to express my deepest appreciation to a number of wonderful people, whom I am greatly indebted to and without whom this thesis would not have been possible.

First and foremost, I am deeply grateful to my research advisor, Prof. Murukeshan Vadakke Matham, for giving me the opportunity to work on this thesis. Prof. Murukeshan has always been very patient and he has provided many valuable advices on research-related matters on numerous occasions in the last four years. I see in Prof. Murukeshan, his passion for research, attention to his research students' progress and personal well-being, and his dedication to deliver what have been promised and many others. All these motivate me to strive hard and also teach me many valuable life-lessons.

Also, special thanks to Mr Ang Teck Meng and Ms Ong Pek Loon. They have always being very helpful and I enjoy working with them. Their technical support rendered to me is very much appreciated.

I am also grateful to every member in the research group who has worked with me. The sharing of research and personal experiences during our conversations has given me new insights in many aspects. I have a fruitful time working with them and would like to thank them for their help and their generosity in sharing with me their experiences and insights.

I would like to express my heartfelt gratitude towards my parents and siblings, who have been a source of inspiration and encouragement to me throughout my life. I am so grateful to them for their relentless support given to me to pursue my ambition and realise my potential. Thanks to my dear wife, a special thank you for your caring and emotional

Acknowledgements

support as I play a new role of a husband to the competing demands of work, research and personal development.

Finally, I would like to thank all those whom I have not specifically mentioned. Your contributions, both big and small, certainly have not gone unnoticed and are also much appreciated.

Abstract

Bio-imaging is of paramount importance in modern medical practices which can be used to acquire unique characteristics of diseases in their early stages so that medical diagnosis and treatment can begin early. This can lead to a better prognosis rate thereby offering potential possibilities for saving many lives. Also, early diagnosis of the diseases can help reduce cost and improve the quality of life. Two diseases, which have been at the forefront of researchers in the recent past due to their probability of cure if detected early, are colon cancer and uveal melanoma.

This thesis in this context aims to investigate the potential of two main imaging modalities, hyperspectral imaging and photoacoustic imaging, individually or by hybrid approach for disease diagnosis. The main objectives of this research thesis are hence directed towards the research and development of novel concepts and methodologies using hyperspectral imaging and photoacoustic imaging for diagnostic bio-imaging applications related to colon cancer and uveal melanoma, respectively.

As part of the thesis, initially a pushbroom hyperspectral imager, which incorporates a video camera for direct video imaging and also for user-selectable region of interest within the field of view of the video camera, has been proposed and successfully demonstrated. The benefits of having user-selectable region of interest include no unwanted scanning and minimal data acquisition time. The system has a spectral range covering the visible to near-infrared wavelength band from 400 nm - 1000 nm and detects 756 spectral bands within this spectral range. This is the main hyperspectral imaging platform to detect cancer progression of different stages inside the colon using the flexible probe-based imaging scheme.

A pushbroom hyperspectral imaging probe based on spatial-scanning method was conceptualised and developed for the first time. The imaging probe is an assembly of a gradient index lens and an imaging fiber optic bundle. The lateral resolutions along the horizontal and vertical directions at 505 nm are about 40 μm . The scope of existing table-top pushbroom hyperspectral imager was extended by enabling it to perform endoscopic bio-imaging using a flexible imaging probe. The pushbroom hyperspectral imaging probe can be used to image the colon for the detection of cancer progression of different stages, while it is generally difficult to access using the conventional table-top systems.

A snapshot hyperspectral video-endoscope is developed using a custom-fabricated two-dimensional to one-dimensional fiber bundle. It converts a pushbroom hyperspectral imager into a snapshot configuration. The fiber bundle is flexible and has a small distal end, enabling it to be used as an imaging probe. It can be inserted into the colon for minimally invasive and *in vivo* investigations for the detection of cancer. The three-dimensional datacubes can provide vast amount of information, which includes the spatial features (shape and size), spectral signatures, speed and direction of the imaged samples.

A hyperspectral photoacoustic spectroscopy system to acquire the normalised optical absorption coefficient spectrum of highly-absorbing bio-samples is also proposed and developed. This allows the characterisation of healthy iris and uveal melanoma in the iris using photoacoustic method, which can be used to detect diseases. Such characterisation is important to determine the optimal wavelength for photoacoustic excitation to have a good contrast between healthy iris and uveal melanoma. Using an optical absorption coefficient reference removes the need to perform spectral calibrations for the wavelength-dependent optical components between the photodiode and the sample.

A probe-based hybrid-modality imaging system was configured and its feasibility was demonstrated with enucleated porcine eye samples. This system is based on a commercial clinical ultrasound imaging platform with a clinical-style imaging probe and a tunable nanosecond pulsed laser. The integrated system uses photoacoustic imaging and ultrasound imaging to provide complementary absorption and structural information, respectively. Photoacoustic and ultrasound B-mode image are acquired at the rate of 10 Hz and about 40 Hz, respectively. Gold nanocages are used as photoacoustic contrast agents, which represent bioconjugated gold nanocages with specific binding, to detect uveal melanoma in the iris. The photoacoustic signals from the iris become stronger after introducing gold nanocages, which can potentially be used as an indication of the location and size of uveal melanoma.

It is envisaged that the major findings and original contributions of this thesis can contribute well towards diagnostic bio-imaging applications pertaining to colon cancer and uveal melanoma.

Table of contents

	Page
Acknowledgements	i
Abstract.....	iii
Table of contents	vi
List of figures.....	xiii
List of tables	xxi
List of symbols.....	xxii
List of abbreviations	xxv
Chapter 1: Introduction	1
1.1 Background and motivation	1
1.1.1 Colon cancer	3
1.1.2 Uveal melanoma	6
1.2 Limitations of current imaging procedures	7
1.3 Objectives	9
1.4 Scope	10
1.5 Organisation of thesis	12
Chapter 2: Literature review	17
2.1 Current medical imaging modalities	17
2.1.1 Medical imaging using ionising radiation.....	18
2.1.1.1 X-ray imaging.....	18
2.1.1.2 Single-photon emission computed tomography (SPECT).....	19
2.1.1.3 Positron emission tomography (PET).....	20
2.1.2 Medical imaging using non-ionising radiation	20
2.1.2.1 Optical imaging	21

Table of contents

2.1.2.2	Ultrasound imaging (USI)	22
2.1.2.3	Magnetic resonance imaging (MRI)	24
2.2	Hyperspectral imaging (HSI)	25
2.2.1	Classification of spectral imaging	26
2.2.2	Datacube	27
2.2.3	Major embodiments of table-top/field HSI	28
2.2.3.1	Spatial-scanning imager	28
2.2.3.2	Spectral-scanning imager	30
2.2.3.3	Snapshot imager	32
2.2.4	Major embodiments of endoscopic HSI	33
2.2.4.1	Spectral-scanning imager	34
2.2.4.2	Snapshot imager	35
2.2.5	Contrast agents (CAs) used in HSI	36
2.2.5.1	Endogenous CAs	36
2.2.5.2	Exogenous CAs	38
2.3	Photoacoustic imaging (PAI)	39
2.3.1	Working principle	40
2.3.2	Major embodiments of PAI	41
2.3.2.1	PA microscopy (PAM)	42
2.3.2.2	PA computed tomography (PACT)	43
2.3.2.3	PA endoscopy	44
2.3.3	Theory	45
2.3.4	Point-illumination PAI using single-element unfocused UST	48
2.3.5	Contrast agents (CAs) used in PAI	50
2.3.5.1	Endogenous CAs	50
2.3.5.2	Exogenous CAs	53
2.4	Overview of imaging modalities mentioned	56
2.4.1	Endoscopic HSI for colon imaging	58
2.4.2	PAI for ocular imaging	60

2.4.2.1	Hybrid-modality imaging	62
Chapter 3: Pushbroom hyperspectral imaging system with selectable region of interest 65		
3.1	Introduction	65
3.2	Instrumentation of pushbroom HSI system.....	66
3.3	Operating principle.....	68
3.4	Calibrations of pushbroom HSI system	69
3.4.1	FOV calibration.....	69
3.4.2	Spectral calibration	70
3.4.3	Position calibration	71
3.4.3.1	Cal _L and Cal _R	71
3.4.3.2	Cal _{LOV}	72
3.5	User-defined parameters.....	73
3.5.1	Region of interest	74
3.5.2	Spectral range.....	74
3.5.3	Stage step size	75
3.5.4	Settings of detector camera	75
3.6	Return values and vectors.....	75
3.6.1	X _{Min} and X _{Max}	75
3.6.2	WL vector	76
3.6.3	Y _{Min} and Y _{Max}	76
3.6.4	Stage position vector.....	77
3.6.5	Significance of return values and vectors	79
3.7	HyperSpec	79
3.8	Data processing and visualization	81
3.9	Results and discussion.....	82
3.9.1	Video camera for selectable ROI	82
3.9.2	Lateral resolution	85

3.9.3	Spectral resolution.....	86
3.9.4	Reflection imaging of bio-sample.....	87
3.9.5	Fluorescence imaging of phantom tissue sample.....	88
3.10	Summary	90
Chapter 4: Pushbroom hyperspectral imaging probe for bio-imaging applications.....		93
4.1	Introduction	93
4.2	Instrumentation of pushbroom HSI probe.....	94
4.3	HyperSpec	96
4.4	GRIN lens.....	96
4.5	Data processing	100
4.6	Results and discussion.....	101
4.6.1	Scale and orientation.....	101
4.6.2	Effective FOV	102
4.6.3	Lateral resolution	102
4.6.4	Reflectance imaging of bio-sample.....	104
4.7	Summary.....	107
Chapter 5: A four-dimensional snapshot hyperspectral video-endoscope for bio-imaging applications.....		109
5.1	Introduction	109
5.2	Instrumentation of HS video-endoscope	110
5.3	Operating principle.....	113
5.4	Spatial calibrations of 2-D to 1-D fiber bundle.....	114
5.4.1	Spatial calibration on 1-D end	114
5.4.2	Spatial calibration on 2-D end	115
5.5	Preparation of bio- and phantom tissue samples.....	115
5.6	Data acquisition.....	116

5.7	Data processing and visualization	116
5.8	Results and discussion	118
5.8.1	Lateral resolution	118
5.8.2	Reflectance imaging of phantom tissue sample	122
5.8.3	Reflectance imaging of bio-sample	125
5.8.4	Fluorescence imaging of phantom tissue sample	128
5.9	Summary	133
Chapter 6: Hyperspectral photoacoustic spectroscopy of highly-absorbing bio-samples		136
6.1	Introduction	136
6.2	Theory	138
6.3	Instrumentation of HS-PAS	140
6.4	Preparation of porcine eye sample	142
6.5	Data processing	142
6.6	Results and discussion	143
6.6.1	Normalised OAC spectrum of OAC reference	144
6.6.2	Validation using fluorescent microsphere suspensions	145
6.6.3	Experiments using enucleated porcine eye samples	147
6.6.3.1	HS-PAS of iris of enucleated porcine eye sample	147
6.6.3.2	Multispectral PA imaging of enucleated porcine eye sample	148
6.6.3.3	Adherence to guideline on exposure limit to laser radiation	150
6.7	Summary	152
Chapter 7: Hybrid-modality ocular imaging using clinical ultrasound system and nanosecond pulsed laser		154
7.1	Introduction	154
7.2	Instrumentation of hybrid-modality ocular imaging system	155
7.3	Preparation of porcine eye samples	157

7.4	Results and discussion.....	158
7.4.1	Spatial resolution.....	158
7.4.2	Imaging of porcine eye samples.....	160
7.4.2.1	Long illumination	160
7.4.2.2	Short illumination for constant fluence	162
7.4.2.3	Reproducible experimental results	165
7.4.2.4	Adherence to guideline on exposure limit to laser radiation	165
7.4.3	Imaging of porcine eye samples with gold nanocages as contrast agent	166
7.5	Summary.....	171
Chapter 8: Conclusions and recommendations for future work.....		173
8.1	Conclusions	173
8.2	Major contributions	177
8.3	Recommendations for future work.....	179
Appendices.....		184
Appendix A: MATLAB® script to arrange two-dimensional data to three-dimensional datacube		185
Appendix B: MATLAB® script to plot cut-datacube.....		187
Appendix C: Spot diagrams using gradient index lens at optimised object-lens distance		189
Appendix D: LabVIEW® software for photoacoustic experiments		191
Appendix E: Adherence to guideline on exposure limit to laser radiation		192
Appendix F: WinProbe ultrasound imaging system.....		195
Appendix G: Synthesis and characterisation of gold nanocages.....		197
Appendix H: Initial photoacoustic experiments using gold nanocages		201
Appendix I: Preparation of porcine eye sample for injection of gold nanocage solution		207
Appendix J: Hyperspectral imaging to authenticate polymer banknotes		208

Table of contents

List of publications	216
References.....	218

List of figures

	Page
Fig. 1.1: Growth curve of solid tumour and its relationship to cancer detection [7].	3
Fig. 1.2: Structure of normal colon [11].	5
Fig. 1.3: Schematic diagram of the eye [14].	6
Fig. 1.4: Uveal melanoma in the iris [17].	7
Fig. 1.5: Research roadmap.....	11
Fig. 2.1: Precession as seen in (a) non-zero spin nuclei in external magnetic field and in (b) spinning top in gravitational field [38].	24
Fig. 2.2: 3-D cut-datacube [52].	27
Fig. 2.3: Data acquired in each scan by different HS imagers [53].	28
Fig. 2.4: Typical table-top pushbroom HS imager [61].	30
Fig. 2.5: Schematic of AOTF [52].	31
Fig. 2.6: Types of reformatter in integral field spectroscopy: (a) fiber bundle, (b) box and (c) rod [73,74].	33
Fig. 2.7: Integral field spectroscopy HS imager using fiber bundle reformatter [53].	33
Fig. 2.8: Concept of image mapping spectroscopy [21].	35
Fig. 2.9: HS endoscope using image mapping spectroscopy [21].	36
Fig. 2.10: (a) Expert labelling and (b) results of HSI after data analysis [63].	37
Fig. 2.11: (a) ROI and (b) blood sO ₂ mapping of retinal vasculature [68].	38

List of figures

Fig. 2.12: (a) ROI and (b) K-means classification overlays under white-light [83].	38
Fig. 2.13: ROI and acquired spectra from selected spatial pixels [54].	39
Fig. 2.14: Forward mode PAI [95].	41
Fig. 2.15: Configurations of (a) OR- and (b) AR-PAM [91].	42
Fig. 2.16: Configurations of PACT using (a) linear- and (b) circular-array UST [91].	43
Fig. 2.17: Side-fire scanning PA endoscope [99].	44
Fig. 2.18: Snapshot PA endoscope [100].	45
Fig. 2.19: PAI of colorectal cancer tissue [100].	51
Fig. 2.20: PAI showing distributions of (a) HbT and (b) blood sO ₂ [109].	52
Fig. 2.21: PAI of lipids [114].	52
Fig. 2.22: PAI of melanin [92].	53
Fig. 2.23: PAI of macrophages loaded with gold NP [108].	54
Fig. 2.24: PA image of Evans blue dye, supplementary notes of [109].	55
Fig. 2.25: PA image indicating the location of injected fluorescent dye [123].	55
Fig. 3.1: Schematic diagram of pushbroom HSI system.	67
Fig. 3.2: Photograph and detailed schematic diagram of pushbroom HSI system.	69
Fig. 3.3: Image from detector camera during spectral calibration of 700 nm.	70
Fig. 3.4: Definition of Cal _L and Cal _R .	72
Fig. 3.5: Cal _L calibration.	72

List of figures

Fig. 3.6: Definition of Cal_{LOV}	73
Fig. 3.7: Cal_{LOV} calibration.	73
Fig. 3.8: Definition of “top, bottom, left and right.”	74
Fig. 3.9: Definition of X_{Min} and X_{Max}	76
Fig. 3.10: Positions of y-axis stage and ROI as scanning progresses.	78
Fig. 3.11: HyperSpec control panel.	80
Fig. 3.12: HyperSpec software protocol.	81
Fig. 3.13: (a) Sequence of data acquisition and (b) datacube.	83
Fig. 3.14: (a) Cut-datacube and (b) wavelength stack of bands 550:25:750 nm.	83
Fig. 3.15: Intensity mappings of nine selected spectral bands.	84
Fig. 3.16: Comparison of ROI and intensity mappings.	85
Fig. 3.17: (a) ROI and (b) intensity mapping of 650 nm.	86
Fig. 3.18: Spectra of 633-nm and 785-nm wavelength sources.	86
Fig. 3.19: (a) Chicken breast tissue on glass slide and (b) ROI.	87
Fig. 3.20: Intensity mappings at (a) 550 nm, (b) 630 nm, (c) 670 nm, and (d) 850 nm.	88
Fig. 3.21: Spectra of blood clot and chicken breast tissue.	88
Fig. 3.22: (a) Rhodamine 6G fluorescent film on tissue phantom and (b) ROI.	89
Fig. 3.23: Intensity mappings of (a) 535 nm, (b) 563 nm (peak emission), and (c) 585 nm.	89
Fig. 3.24: Normalised excitation and fluorescence spectra.	89

List of figures

Fig. 4.1: Schematic diagram of pushbroom HSI probe.....	95
Fig. 4.2: Optimised layout of GRIN lens at five representative wavelengths.	98
Fig. 4.3: Zemax spot diagram of 550 nm on distal end-face of fiber bundle.....	98
Fig. 4.4: Zemax spot diagram of 1000 nm on distal end-face of fiber bundle.....	99
Fig. 4.5: Comparison of ROI and intensity mappings of USAF chart G2E4.	101
Fig. 4.6: (a) ROI and (b) intensity mapping of horizontal bars of USAF chart G1E6.....	102
Fig. 4.7: Images of USAF chart Group 3. ROIs of (a) G3E1 and G3E2, (b) G3E3 and G3E4, (c) G3E5 and G3E6, 505-nm intensity mappings of (d) G3E1 and G3E2, (e) G3E3 and G3E4, and (f) G3E5 and G3E6.	103
Fig. 4.8: Nine selected intensity mappings of USAF chart G3E5 and G3E6.	104
Fig. 4.9: (a) Sample of chicken breast tissue with blood clot and (b) ROI.....	104
Fig. 4.10: Cut-datacube of chicken breast tissue with blood clot.	105
Fig. 4.11: Four selected intensity mappings of chicken breast tissue with blood clot.....	106
Fig. 4.12: Mean reflectance spectra (white lines) and standard deviation (black areas) of chicken breast tissue and blood clot.....	106
Fig. 5.1: Instrumentation of snapshot HS video-endoscope.	112
Fig. 5.2: Photograph of 2-D to 1-D fiber bundle.	112
Fig. 5.3: Photograph of (a) 2-D and (b) 1-D end-faces showing all fiberlets.	113
Fig. 5.4: Reference image taken by detector camera.	114

List of figures

Fig. 5.5: (a) Photograph and (b) digital mask of fiberlets on 2-D end-face.....	115
Fig. 5.6: Imaged regions of USAF chart (a) G1E5 and (b) G2E3.	119
Fig. 5.7: Transmittance mappings of nine datacubes of G1E5 at 500 nm.	120
Fig. 5.8: Transmittance mappings of nine datacubes of G2E3 at 500 nm.	121
Fig. 5.9: (a) Simulated phantom tissue sample and (b) photograph of the 2-D end of fiber bundle superimposed on the imaged region of sample.	122
Fig. 5.10: Cut-datacubes acquired using frames (a) 21, (b) 35 and (c) 44.....	123
Fig. 5.11: 4-D reflectance mappings of nine selected wavelengths and datacubes.	124
Fig. 5.12: Mean reflectance spectra with standard deviations of Regions R1 and R2.....	125
Fig. 5.13: (a) Bio-sample and (b) photograph of the 2-D end of fiber bundle superimposed on sample.	126
Fig. 5.14: Reflectance mappings of nine datacubes at 600 nm.....	127
Fig. 5.15: Mean reflectance spectra with standard deviations of Regions B1, B2 and B3.	128
Fig. 5.16: (a) Simulated phantom tissue sample and (b) photograph of the 2-D end of fiber bundle superimposed on sample.	129
Fig. 5.17: Cut-datacubes acquired using frames (a) 18, (b) 58 and (c) 128.....	130
Fig. 5.18: Fluorescence mappings of nine datacubes at 585 nm.....	131
Fig. 5.19: Mean fluorescence spectra with standard deviations of Regions F1, F2 and F3.....	132
Fig. 6.1: Schematic diagrams of HS-PAS setup for (a) measurement with eye and OAC reference and (b) validation.	141

Fig. 6.2: (a) UST and (b) photodiode signals of OAC reference using 500-nm excitation.	143
Fig. 6.3: (a) $P_V(\lambda)$ and (b) $F_V(\lambda)$ of the OAC reference and sample.	143
Fig. 6.4: (a) Assumed behaviour of light in OAC reference, experimental setup to measure (b) transmittance and (c) reflectance of OAC reference.	145
Fig. 6.5: Normalised OAC spectrum of reference $\mu_{Ref_N}(\lambda)$	145
Fig. 6.6: $\mu_{Sam_N}(\lambda)$ of Red fluorescent microsphere suspension.	146
Fig. 6.7: Validation results using (a) Red, (b) Crimson and (c) Nile Red fluorescent microsphere suspensions.	147
Fig. 6.8: Measured normalised OAC spectrum of iris in porcine eye sample.	147
Fig. 6.9: (a) Schematic of the eye, B-scan images across the centre of the eye using (b) 465 nm (c) 750 nm and (d) 870 nm.	149
Fig. 6.10: Schematic of laser beam exiting objective lens 2.	151
Fig. 7.1: Instrumentation of hybrid-modality imaging system.	156
Fig. 7.2: (a) PA and (b) US images of human hair.	159
Fig. 7.3: Normalised Gaussian fittings of axial and lateral profiles of (a) PA and (b) US images of human hair.	160
Fig. 7.4: (a) Schematic diagram of eye and (b) US image of porcine eye sample.	161
Fig. 7.5: (a) PA and (b) combined PA/US images of enucleated porcine eye sample.	162
Fig. 7.6: (a) PA and (b) combined images with lens illumination, and (c) PA and (d) combined images with iris illumination.	163

List of figures

Fig. 7.7: (a), (b), (c) and (d) are four sets of combined images from porcine eye samples.	165
Fig. 7.8: Combined images of porcine eye sample A (a) before and (b) after injection of AuNcg solution.	169
Fig. 7.9: Combined images of porcine eye sample B (a) before and (b) after injection of AuNcg solution.	169
Fig. 7.10: Combined images of porcine eye sample C (a) before and (b) after injection of AuNcg solution.	170
Fig. 7.11: Combined images of porcine eye sample D (a) before and (b) after injection of AuNcg solution.	170
Fig. 7.12: Increase in strength of PA signals after injection of AuNcg solution.	171
Fig. 8.1: Beam splitter for delivery of illumination.	181
Fig. 8.2: Improved two-dimensional to one-dimensional fiber bundle probe showing front-views of all ends.	182
Fig. 8.3: Side-view of distal end of improved fiber bundle probe.	182
Fig. C.1: Zemax spot diagram of 400 nm on distal end-face of fiber bundle.	189
Fig. C.2: Zemax spot diagram of 700 nm on distal end-face of fiber bundle.	190
Fig. C.3: Zemax spot diagram of 850 nm on distal end-face of fiber bundle.	190
Fig. D.1: Control panel of developed LabVIEW® software.	191
Fig. F.1: Photograph of WinProbe scanner shown with ultrasound transducers used.	195
Fig. F.2: Control panel of UltraVision software.	195

Fig. F.3: (a) L15 and (b) L8 clinical ultrasound transducers from WinProbe.	196
Fig. G.1: (a) TEM image of AuNcg with inset showing the FFT image, (b) zoom-in of one corner of AuNcg, (c) line profile of FFT image in (a), and (d) line profile of TEM image of AuNcg shown in (b).	199
Fig. G.2: (a) SEM and (a) inverted greyscale SEM images of AuNcgs.	200
Fig. G.3: Ultraviolet-visible absorbance spectra of AgNcbs and AuNcgs.	200
Fig. H.1: Processed signals of four selected AuNcgs concentrations.	202
Fig. H.2: P_{Max} against AuNcg concentration.	203
Fig. H.3: (a) Three tubings held in place by acrylic holder and (b) close-up of tubings. ...	204
Fig. H.4: Combined PA/US images of excited (a) left, (b) centre and (c) right tubings. ...	205
Fig. I.1: Injection of gold nanocage solution above left iris of porcine eye sample.	207
Fig. J.1: Locations and ROIs of (a) Lion, (b) Dot, (c) Number and (d) Cap of RefNote1.	210
Fig. J.2: Cut-daticubes of (a) Dot and (b) Number of measurement 1 of RefNote1.	210
Fig. J.3: $\#$ Reflectance spectra from reference banknotes of (a) Lion, (b) Dot, (c) Number and (d) Cap.	211
Fig. J.4: ROIs of (a) Lion, (b) Dot, (c) Number and (d) Cap of CF1.	212
Fig. J.5: $\wedge$ Reflectance spectra from genuine banknotes and reference spectra of a) Lion, b) Dot, c) Number and d) Cap.	213
Fig. J.6: $\ast$ Reflectance spectra from simulated counterfeit banknotes and reference spectra of a) Lion, b) Dot, c) Number and d) Cap.	214

List of tables

	Page
Table 2.1: Classification of spectral imaging.....	26
Table 2.2: Summary of ionising biomedical imaging modalities.	56
Table 2.3: Summary of non-ionising biomedical imaging modalities.....	57
Table 2.4: Comparison between conventional optical imaging methods and HSI for colon cancer detection.....	59
Table 2.5: Comparison between conventional imaging methods and hybrid-modality imaging for uveal melanoma detection.	64
Table 6.1: Selected wavelengths and measured pulse energy.	152
Table 7.1: Parameters for calculations of repetitive pulse exposures ^a	166
Table E.1: Parameters for calculations of repetitive pulse exposures ^a	192
Table E.2: EL_1 and $Ratio_1$	193
Table E.3: $EL_{2,A}$ and $Ratio_{2,A}$	193
Table E.4: $EL_{2,B}$	194
Table J.1: Summary of reflectance RMSE (%).	214

List of symbols

Symbol	Description
β	Thermal coefficient of volume expansion
ε	Molar absorption
η_{th}	Percentage energy converted to heat
Γ	Grüneisen parameter
λ	Wavelength
μ	Optical absorption coefficient
Φ	Optical fluence rate
θ	Angular subtense
a	Spectral calibration constant
b	Spectral calibration constant
“Bottom”	Row index of video camera’s sensor array which corresponds to the bottom of region of interest
c	Spectral calibration constant
C_A	Spectral correlation factor
C_P	Isobaric specific heat capacity
Cal_{FOV}	Length of field of view of video camera in vertical direction
Cal_{LOV}	Row index of video camera’s sensor array which shares same view as line of view of detector camera
Cal_L, Cal_R	Column indexes of detector camera’s sensor array which correspond to extreme left and right views of video camera, respectively
CD	Count-displacement relationship of y-axis stage
Conc	Concentration
DC_X, DC_Y	Column and row indexes of detector camera’s sensor array, respectively
EL_1	Energy exposure limit for single pulse
EL_2	Energy exposure limit for repetitive pulses
EL_{Rep}	Exposure limit for repetitive pulses
EL_{SP}	Exposure limit for single pulse
F	Optical fluence

List of symbols

Symbol	Description
F_{PD}	Signals acquired by photodiode after taking into account its responsivity
$F_{PD,raw}$	Signals acquired by photodiode
F_V	Area under photodiode signals
H	Heating function
I	Light intensity
I_0	Incident light intensity
L	Length (thickness)
“Left”	Column index of video camera’s sensor array which corresponds to the left of region of interest
n	Refractive index
P	Acoustic pressure
P_0	Initial acoustic pressure
P_{Max}	Maximum amplitude of signals acquired by ultrasound transducer after Hilbert transformation, fluence variation compensation and background signal correction
P_{UST}	Signals acquired by ultrasound transducer after undergoing Hilbert transformation
$P_{UST,raw}$	Signals acquired by ultrasound transducer
P_V	Maximum amplitude of signals acquired by ultrasound transducer after Hilbert transformation
Pos_{End}	Position of y-axis stage for final scan (counts)
Pos_{Start}	Position of y-axis stage for first scan (counts)
QE	Quantum efficiency of detector camera
$\mathbf{r}$	Position
r_1	Radius of laser beam exiting objective lens 2
r_2	Radius of laser spot on sample
R	Reflectance
$Resp$	Responsivity of photodiode
“Right”	Column index of video camera’s sensor array which corresponds to the right of region of interest
“Step”	User-defined step displacement of y-axis stage (distance imaged by certain number of rows of video camera’s sensor array)

List of symbols

Symbol	Description
Step _{Cts}	Step displacement of y-axis stage (counts)
t	Time
t_{Pulse}	Pulse duration
T	Transmittance
T _{Train}	Exposure duration for each wavelength
T _{Max}	Total exposure duration
Temp	Temperature
“Top”	Row index of video camera’s sensor array which corresponds to the top of region of interest
v_s	Speed of sound in medium
VC _X , VC _Y	Column and row indexes of video camera’s sensor array, respectively
WL	Wavelength assigned to each row of detector camera’s sensor array
WL _{Cal}	Calibration wavelength
WL _{Min} , WL _{Max}	User-defined lower and upper bounds of spectral range for data acquisition
x	Spatial dimension
X _{Length}	Number of column of detector camera’s sensor array for data acquisition
X _{Min} , X _{Max}	Column indexes of detector camera’s sensor array which correspond to the “Left and Right” of region of interest, respectively
y	Spatial dimension
Y _{Length}	Number of row of detector camera’s sensor array for data acquisition
Y _{Min} , Y _{Max}	Row indexes of detector camera’s sensor array which correspond to WL _{Min} and WL _{Max} , respectively
Y _{Pos}	Current y-axis stage position (counts)
z	Spatial dimension
Subscript	
N	Normalised
Ref	Reference
Sam	Sample

List of abbreviations

Abbreviation	Explanation
1-D	One-dimensional
2-D	Two-dimensional
3-D	Three-dimensional
4-D	Four-dimensional
AgNcb	Silver nanocube
ALS	Anterior lens surface
AOTF	Acousto-optical tunable filter
AR-PAM	Acoustic-resolution photoacoustic microscopy
AuNcg	Gold nanocage
CA	Contrast agent
EL	Exposure limit
EM	Electron-multiplying
FFT	Fast Fourier transform
FOV	Field of view
G1E5	Group 1 Element 5
G2E4	Group 2 Element 4
G3E5	Group 3 Element 5
GRIN	Gradient index
HbO ₂	Oxy-haemoglobin
HbR	Deoxy-haemoglobin
HbT	Total haemoglobin concentration
HS	Hyperspectral
HSI	Hyperspectral imaging
HS-PAS	Hyperspectral photoacoustic spectroscopy
LCTF	Liquid crystal tunable filter
LOV	Line of view
MRI	Magnetic resonance imaging

List of abbreviations

Abbreviation	Explanation
NA	Numerical aperture
NIR	Near-infrared
NP	Nanoparticle
OAC	Optical absorption coefficient
OCT	Optical coherence tomography
OR-PAM	Optical-resolution photoacoustic microscopy
PA	Photoacoustic
PACT	Photoacoustic computed tomography
PAI	Photoacoustic imaging
PAM	Photoacoustic microscopy
PET	Positron emission tomography
PVP	Polyvinylpyrrolidone
PRF	Pulse repetition frequency
RMSE	Root-mean-square error
RMSE _{Aut}	Root-mean-square error for authentication
ROI	Region of interest
SEM	Scanning electron microscopy
sO ₂	Oxygen saturation
SPECT	Single-photon emission computed tomography
TEM	Transmission electron microscopy
US	Ultrasound
USAF	United States Air Force
USI	Ultrasound imaging
UST	Ultrasound transducer

Chapter 1: Introduction

This chapter begins with the background and motivation for embarking upon this challenging research thesis. This will be followed by a brief review on some of the potential diseases in correlation with their diagnostic methodologies which are currently in practice or reported elsewhere in the literature. Diagnostics of the two targeted diseases in this thesis, colon cancer and uveal melanoma, are then discussed in detail. The chapter then focuses on the major objectives of this doctoral research followed by its scope and the drafted research roadmap for achieving the laid out research targets. The chapter concludes with the organisation of the thesis.

1.1 Background and motivation

Medical imaging refers to the concepts and methodologies used to image the body or parts of it for medical diagnostics purposes. It plays a crucial role in the field of medicine because it can highlight the functional and structural changes in the body, which lead to eventual diseases such as cancers and acute coronary events. It is also vital to detect these diseases at their early stages and diagnose medical conditions when patients undergo medical check-up. Some diseases have high morbidity and mortality rates. However, these rates can be greatly reduced with early diagnosis and medical procedures [1,2].

Certain specific abnormalities produced in the early stage of the disease cannot be easily differentiated from the surrounding healthy tissues due to their small size and very similar properties that they exhibit. Under such situations, these abnormalities may prevent detection using normal diagnostic procedures, thus delaying treatment which can deteriorate patient's health and increase the likelihood of death.

Although there are methods and equipment using ionising radiation such as positron emission tomography, single-photon emission computed tomography and other non-optical imaging methods using radioactive materials, they are not preferred for obvious reasons.

Hence imaging methods using non-ionising radiation, such as optical imaging, are heavily preferred for most diagnostic imaging needs. Diseases can occur at many different parts of the body. Some occur directly on the skin and thus relatively easy to access for medical imaging. However, other diseases like colon cancer take place within the body in the gastrointestinal tract. This makes conventional imaging setup unsuitable for non-invasive or minimally-invasive diagnostic applications. As much as possible, medical imaging should be non-invasive so that there is no physical damage to the surrounding tissues or organs during the imaging process.

In this context, the main motivation for pursuing this research thesis is the prevailing situation of disease occurrence and the limitations of the present tools for early disease diagnosis. A good imaging method for diagnosis at the early stages of disease means there is a high chance for a complete cure. Also, the routine procedures should be safe for regular check-ups and has very low or if possible, no risk or any adverse side effects. For certain diagnostic purposes, it should also be capable to image the body from within. A data library of the characteristics of diseases can help clinicians make better diagnostic evaluation and confirmation of diseases. In the case of cancer, such *in vivo* biopsy may one day eliminate the need to do a tissue excision for biopsy [3-5].

Furthermore, early diagnosis of the diseases can help reduce cost and increase the quality of life and reduce mortality rates. From this perspective, the following sections discuss the two targeted diseases in this thesis (colon cancer and uveal melanoma) and highlight the potential problems and limitations of the current imaging and diagnostic procedures.

1.1.1 Colon cancer

Cancer is the second leading cause of death in 2009 in the United States [6]. In 2012, the estimated new cases due to cancers in the digestive system (colon, pancreas), respiratory (lungs), genital system (ovary, prostate) and urinary system (kidney, bladder) stands at about 1 million, and resulted in about 0.4 million death cases. This accounts for more than half of the total estimated new cases and deaths in the United States, and an increasing trend of cancer incidence rate from 1975 to 2008 [1].

Fig. 1.1: Growth curve of solid tumour and its relationship to cancer detection [7].

In the initial stage of cancer growth, tumours of microscopic size have not recruited new blood vessel. Therefore they can only lay less than 200 μm next to existing blood vessels to acquire the needed oxygen and nutrients for long-term survival. This is due to the diffusion limit of oxygen being about 100 μm . This also limits the size of tumours to less than 1 mm, before they are able to recruit new blood vessels [8].

Angiogenic switch refers to the phase in cancer growth where the tumour starts its recruitment of blood vessels (Fig. 1.1). After angiogenic switching, the tumours are able to recruit its own vascular supply and thus expand in size. Further mass expansion will then lead to the tumours becoming clinically detectable [8]. The aim of medical imaging to detect cancer is to image the smallest tumour possible before it undergoes angiogenic

switching [7] to become a highly malignant and deadly phenotype [8]. Remission means the uncertainty in tumour cell size before it can be detected, and this depends on the minimum detection threshold of the imaging method used. Current remission for solid tumours is about 10^9 cells, which have a mass of 1 g or volume of 1 cm^3 [7].

One of the two targeted diseases in this thesis is colon cancer. This form of cancer has the second highest number of estimated new cases and deaths in the United States in 2012 [1]. During the period 2008-2012 in Singapore, colon cancer is the most and second most frequent cancer among the males and females, respectively. It accounts for 17.5% and 13.6% of all cancers among the males and females in Singapore, respectively [9]. This makes colon cancer one of the most frequent cancers in the general population. Within the same period in Singapore, colon cancer is also the second and third leading cause of cancer deaths among the males (1926 counts) and females (1650 counts), respectively [9]. Like many other types of cancer, colon cancer can have better prognosis and higher survival rate when treatment therapies in the early stage of diseases can be conducted. Among the males in Singapore diagnosed with Stage I, II, III and IV colon cancer during 2003-2007, the observed survival rate after five years of diagnosis is 80.7%, 69.3, 51.1% and 7.9%, respectively [10]. Similar trend can be observed among the females. These figures show that the earlier the diagnosis of colon cancer, the higher the observed survival rate. The five years observed survival rate of a male resident diagnosed with Stage I colon cancer is very high (80.7%), and it is about 10 times more than that of another diagnosed with Stage IV colon cancer. It validates the importance of medical imaging capable of early diagnosis of colon cancer.

The colon has four layers, starting from the innermost layer mucosa, which is surrounding the lumen, or the hollow space within the colon. The next layer is the submucosa, followed by the muscle layers and serosa (Fig. 1.2). Each layer is about 0.9 mm thick and thus the thickness of the colon wall is up to 3.6 mm. Like many other types of cancer, colon cancer can be staged. Cancer staging is critical as it will determine both treatment and prognosis. Colon cancer can be classified into five stages, from Stage 0 to Stage IV [11], each with increasing spread of the cancerous cells.

Colon cancer starts off with Stage 0 in the innermost layer of the colon wall (mucosa). This stage is also called carcinoma in situ. Abnormal cells are found in the innermost mucosa and may later become cancer and spread.

In Stage I, the abnormal cells in Stage 0 become cancer in the mucosa and spread further into the second layer of the colon wall (submucosa). Cancer may have spread to the muscle layer of the colon wall.

Fig. 1.2: Structure of normal colon [11].

Stage II colon cancer is divided into Stage IIA, Stage IIB, and Stage IIC. In Stage IIA, cancer spreads through the muscle layer and to the serosa, which is the outermost layer of the colon wall. In Stage IIB, cancer spreads through the serosa but has not spread to nearby organs. In Stage IIC, cancer spreads through the serosa and to nearby organs.

Stage III colon cancer is divided into Stage IIIA, Stage IIIB, and Stage IIIC. Each of these stages can also be made up of a few scenarios. In general, Stage III cancer spreads through the mucosa and submucosa, and may even reach the deeper layers of the colon. Also, at least one nearby lymph node is affected. The main difference between Stage II and III is that the latter have cancers have spread to the nearby lymph nodes.

In Stage IV colon cancer, cancer spreads through the blood and lymph nodes to distant parts or organs of the body. Stage IV colon cancer is divided into Stage IVA and Stage IVB. Colon cancer in Stage IVA spreads to one distant organ or lymph node while in Stage IVB, cancer spreads to more than one distant organ or into the lining of abdominal wall.

1.1.2 Uveal melanoma

Fig. 1.3 shows the structure of an eye and the anterior-to-posterior diameter of a human eyeball is about 24 mm [12]. Vision trouble is defined as having difficulty in seeing, even with the aid of glasses and contact lenses. And it is experienced by close to 10% of the adult population in the United States. Age was also identified as being positively associated with vision trouble [13]. Thus, vision trouble can be a significant problem especially in aging societies such as Singapore.

Fig. 1.3: Schematic diagram of the eye [14].

Vision trouble can be caused by a variety of ocular diseases such as glaucoma and uveal melanoma, a type of intraocular cancer. Uveal melanoma is the most common ocular tumour in older individuals which is found near critical ocular structures, such as the iris (Fig. 1.4), choroid and ciliary body [15]. Without early detection and treatment, it will result in painful eye, loss of vision and in some cases deaths due to metastatic disease [15,16].

Fig. 1.4: Uveal melanoma in the iris [17].

1.2 Limitations of current imaging procedures

A common yet important method to detect early colon cancer is to use white light colonoscopy [18]. An endoscope is used to image the colorectal region directly, and then a clinician tries to identify the lesions in the image. Lesions that are flat, depressed and subtle may be present in the image but not recognised by the clinician, as they are not easily identifiable [19]. This also depends on the clinician's experience and performance. A way to reduce the variations among clinicians' performance is to use chromo-endoscopy (dye spraying), but it is not proven to be better than colonoscopy done by high-performance clinicians [19]. Detecting lesions using colonoscopy and similar methods will to a certain extent be affected by error in human judgement, especially for small lesions with subtle changes.

Hyperspectral imaging records the intensity of narrow and adjacent spectral bands over a large spectral range. This provides spectral signatures to be used for classification and quantification, which can in turn be used to detect diseases. The tissue properties of normal

tissue and tumour are different, resulting in different reflectance and fluorescence properties [20]. Therefore, hyperspectral imaging can be used to find these spectral differences for the detection of colon cancer using both reflectance and fluorescence imaging modalities. Also, the availability of hyperspectral endoscopes makes it suitable for colon imaging [21]. Hyperspectral imaging can be used to create a data library to help clinicians make better diagnostic evaluation and confirmation of diseases. This removes the need for the actual tissue excision as the results can be known on the spot.

Uveal melanoma can be detected using a few imaging methods, such as angiography, ophthalmoscopy and ultrasonography [15]. Although these methods are useful, it can only provide limited information when used on its own which may not be sufficient for a more comprehensive diagnosis of uveal melanoma. Also, the use of angiography may not always be preferred, as it introduces foreign substance such as fluorescein and indocyanine green into the body [15].

Photoacoustic imaging is a relatively new imaging modality with optical excitation and ultrasonic detection. It uses safe non-ionising radiation and thus free from all the radiation risks. The contrast in photoacoustic imaging is due to optical absorption heterogeneity, which differs between normal tissue and tumour. Therefore, photoacoustic imaging can be used to find these differences for the detection of uveal melanoma. Also, photoacoustic imaging can be integrated with ultrasound imaging as both imaging modalities are detecting acoustic waves using an ultrasound transducer. The combination of these two imaging modalities also makes it easier for clinicians to accept photoacoustic imaging as an emerging imaging modality [22]. By doing so, a hybrid-modality imaging which uses imaging modalities of different operation principles can be acquired. This approach

provides complementary and clinically useful information more than what is provided by one imaging modality so that a better diagnostic evaluation and confirmation of uveal melanoma can be made [23].

1.3 Objectives

The limitations of current imaging procedures as well as the potential of hyperspectral and photoacoustic imaging are outlined in the previous section. From these perspectives, the main objectives of this thesis are directed towards the research and development of novel concepts and methodologies using hyperspectral imaging and photoacoustic imaging for diagnostic bio-imaging of the targeted diseases and related applications. These include:

- (i) To design and optically engineer an endoscopic hyperspectral imaging system for imaging in the gastrointestinal tract with a spectral resolution of the order of few nanometres within a wavelength band of 400 nm - 1000 nm.

This is expected to find potential application by way of creating spectral data library for disease diagnosis.

- (ii) Investigation into a probe-based hybrid-modality imaging platform for diagnostic ocular imaging in order to detect uveal melanoma. The targeted specifications of the imaging system are expected to have a spatial resolution of 1 mm and the excitation wavelength should be tunable with a wavelength resolution of 1 nm.

A probe-based hybrid-modality imaging system by integrating photoacoustic imaging and ultrasound imaging is researched here to achieve the targeted objectives.

The proposed research includes the development of novel concepts, relevant theoretical simulations, methodologies, instrumentation and follow-up experimental validations.

1.4 Scope

This section outlines the scope of the research work carried out which has been designed and adopted to meet the above mentioned desired objectives. The research roadmap (Fig. 1.5) summarises the research methodology executed for this thesis.

- (i) Research and development of a table-top pushbroom hyperspectral imager integrated with a video camera for user-defined region of interest using custom-developed software.
- (ii) Conceptualisation, development and experimental demonstration of a probe-based pushbroom hyperspectral imaging system for endoscopic applications. Numerical investigations into how probe lens affects imaging characteristics of system.
- (iii) Design and fabrication of an endoscopic snapshot hyperspectral imaging system suitable for high spectral resolution and real-time applications. Experimental investigations using bio- and fluorescent phantom tissue samples.
- (iv) Conceptualisation and development of a table-top hyperspectral photoacoustic spectroscopy system for bio-samples. Theoretical and experimental investigations on the use of an optical absorption coefficient reference.
- (v) Investigations into a probe-based hybrid-modality imaging system using photoacoustic imaging and ultrasound imaging as a dual-modality imaging in a single platform. Use of plasmonic gold nanocages to enhance contrast in photoacoustic images. Experimental investigations using enucleated porcine eye samples.

Fig. 1.5: Research roadmap.

1.5 Organisation of thesis

This thesis is organised into 8 chapters. Each chapter begins with a short note reflecting the main contents of the chapter.

Chapter 1 is an introductory chapter and it gives an overview about the present status of the problem. The main motivation of the thesis is the prevailing situation of two targeted diseases, namely the colon cancer and uveal melanoma. These diseases are not only prevalent in many parts of the world, but in Singapore as well. It is followed by the objectives and scope of this thesis. A block diagram of the research roadmap is presented, followed by the organisation of the thesis which is given in the last section of this chapter.

Chapter 2 contains a detailed literature review that has been carried out for this thesis, divided into three main sections. Section A discusses the common imaging modalities that are being used in biomedical imaging. This section is broadly divided into two parts, ionizing and non-ionizing imaging. In each part, a few imaging method will be discussed. This is followed by Section B where another two imaging modalities, namely hyperspectral and photoacoustic imaging, are reviewed in details. These two modes of imaging are the main focus of this thesis, thus a lot of emphasis was given to them in this chapter. Section C contains the outcome of this literature review and the need for a multimodality and hybrid-modality imaging.

Chapter 3 presents a novel spatial-scanning pushbroom hyperspectral imaging system incorporating a video camera. Existing hyperspectral imaging systems with a video camera is only used for direct video imaging. However, the system presented in this chapter also uses the video camera for the selection of the region of interest within its field of view. Using a video camera for these two applications brings many benefits to a pushbroom

hyperspectral imaging system, such as a minimal data acquisition time and smaller data storage requirement. A detailed description of the system followed by the methods and formulas used for calibration and electronic hardware interfacing are discussed. This system captures 756 wavelength bands covering the spectral region from visible light to near-infrared (400 nm - 1000 nm). United States Air Force resolution chart, chicken breast tissue, and fluorescent targets are used as test samples. The results from these test samples prove that the various aspects of the system are integrated correctly and are able to capture hyperspectral images of bio-samples in reflection and fluorescence imaging. This is the main hyperspectral imaging platform for probe-based imaging in the colon to detect cancer progression of different stages by integrating it with a flexible probe scheme, as detailed in the next two paragraphs.

Chapter 4 presents a spatial-scanning pushbroom hyperspectral imaging probe, which is the first to employ such spatial-scanning method. The system is realised by integrating a pushbroom hyperspectral imager with an imaging probe. The imaging probe is configured by incorporating a gradient index lens at the end-face of an image fiber bundle. The necessary detailed instrumentation, methodology and theoretical simulations of the gradient index lens that are carried out are explained. This is followed by the assessment of the developed probe's performance. Resolution test targets such as United States Air Force chart as well as bio-samples such as chicken breast tissue with blood clot are used as test samples. The system's imaging characteristics are determined and it is shown that the system can successfully capture hyperspectral bio-images.

Chapter 5 demonstrates a novel four-dimensional snapshot hyperspectral video-endoscope for bio-imaging applications. It has a frame rate of about 6.16 Hz and spectral

range of 400 nm - 1000 nm. It also captures 756 spectral bands which are significantly more than existing snapshot hyperspectral video-endoscopes which can generally capture only about 50 spectral bands. With more spectral bands available, limitations such as a reduced spectral range, insensitivity to certain narrow spectral band and inability to capture detailed spectral signatures, can be avoided. Capturing the three-dimensional datacube sequentially gives the fourth dimension. All these are achieved by using a custom-designed and fabricated compact biomedical probe, which converts a table-top pushbroom hyperspectral imager into an endoscopic snapshot configuration. The fiber bundle is flexible and has a small distal end enabling it to be used as an imaging probe that can be inserted into the colon for minimally invasive and *in vivo* investigations for the detection of cancer. The detailed instrumentation of the proposed system is presented. The lateral resolutions of the system along the horizontal and vertical directions are found to be 157.49 μm and 99.21 μm , respectively. The feasibility of the proposed system is demonstrated by imaging bio- and phantom tissue samples representing different stages of cancer growth in reflectance and fluorescence imaging modalities.

Chapter 6 proposes and illustrates a hyperspectral photoacoustic spectroscopy system to measure the absorption-related properties of highly-absorbing samples directly. This allows the characterisation of healthy iris and uveal melanoma in the iris using photoacoustic method, which can be used to detect diseases. Such characterisation is important to determine the optimal wavelength for photoacoustic excitation such that there is good contrast difference between healthy iris and uveal melanoma. The system in this chapter measures using 461 wavelength bands instead of the tens of wavelength bands used in other reported photoacoustic spectroscopy. The use of an optical absorption coefficient reference

is also proposed to remove the need to perform spectral calibration to account for the wavelength-dependent transmittance and reflectance of the optical components used in the setup. The normalised optical absorption coefficient spectrum of the highly-absorbing iris of enucleated porcine eye sample is acquired. The proposed concepts and the feasibility of the developed system are demonstrated by using fluorescent microsphere suspensions and porcine eyes as test samples.

Chapter 7 presents a hybrid-modality imaging system based on a commercial clinical ultrasound imaging system using a linear-array ultrasound transducer and a tunable nanosecond pulsed laser to provide optical excitation for ocular imaging. The integrated system uses photoacoustic and ultrasound imaging to provide complementary absorption and structural information of the eye. In this system, B-mode images from photoacoustic and ultrasound imaging are acquired at 10 Hz and about 40 Hz, respectively. A linear-array ultrasound transducer makes the system of a snapshot configuration, compared to other ocular imaging systems using a single-element ultrasound transducer which require scanning to form B-mode images. The results show that the proposed instrumentation is able to incorporate photoacoustic and ultrasound imaging in a single setting. The feasibility and efficiency of this developed probe system is illustrated by using enucleated porcine eyes as test samples. It is demonstrated that photoacoustic imaging could capture photoacoustic signals from the iris, anterior lens surface, and posterior pole, while ultrasound imaging could accomplish the mapping of the eye to reveal the structures like the cornea, anterior chamber, lens, iris, and posterior pole. Hybrid-modality imaging of the eye can provide complementary and clinically useful information, so that a better diagnostic evaluation and confirmation of uveal melanoma can be made by clinicians. Gold nanocages are used as

photoacoustic contrast agents, which represent bioconjugated gold nanocages with specific binding to detect uveal melanoma in the iris. Photoacoustic images are taken from enucleated porcine eye samples before and after the introduction of gold nanocage solution above the iris. The photoacoustic signals from the iris become stronger after gold nanocages are introduced, which can potentially be used as an indication of the location and size of uveal melanoma.

Chapter 8 is the last chapter of this thesis. It begins with the conclusions and highlights the major contributions of this thesis. This is followed by the recommendations for future work directions.

Chapter 2: Literature review

This literature review chapter is divided into three main sections, A, B and C. Section A discusses the common types of imaging modality that have been used in clinician environment. A few imaging methods in both the ionizing and non-ionizing imaging methods are included. Section B covers the two imaging modalities that are selected for this thesis as the main focus. The first imaging modality is hyperspectral imaging. Its definition used in this thesis, the data acquired, major embodiments in table-top/field and endoscopic applications and contrast agents are discussed. The second is photoacoustic imaging where the working principle, major embodiments, theory and contrast agents are discussed. Also included is how experimental data are acquired and processed to produce the photoacoustic image. Section C, the last section of this thesis discusses the outcome of the literature review. The need for a multi or hybrid-modality imaging is also discussed.

Section A: Current imaging modality

2.1 Current medical imaging modalities

Modern medical imaging plays a vital role in the field of medicine as they can be used for clinical diagnosis applications. Over the years, several types of medical imaging modalities have been developed for different applications. These imaging modalities have different characteristics and their own benefits and limitations. They can be broadly divided into two main categories, those with and without the use of ionising radiation. The key difference between these two categories is the type of rays or waves used for imaging, and thus the radiation effect it brings along for those using ionising radiation.

Contrast agent (CA) provides the contrast in biomedical images and there are two types, endogenous and exogenous CA. Endogenous CA exists naturally within the body and its presence creates intensity differences in biomedical images to form a representation of the imaged region. On the other hand, an exogenous CA is a substance that is not present in the body and has to be administered into the body for the same purpose. Some substances or

tissues in the body can be imaged directly to provide such contrast. However, exogenous CAs can still be used to further enhance the contrast to form images of higher quality.

2.1.1 Medical imaging using ionising radiation

Medical imaging using ionising radiation makes use of high-frequency and high-energy waves in the electromagnetic spectrum, such as X-ray and Gamma-ray. Ionising radiation has sufficient energy to free electrons from atoms and molecules, causing ionisation of the tissues which can lead to tissue damage [24]. The side-effects of radiation include cell death and higher risk of cancer [25]. These inherent radiation risks exist even under the exposure of low-dose ionising radiation [26]. However in most cases, the risk is small compared to the benefits provided by such medical imaging modalities using ionising radiation.

2.1.1.1 X-ray imaging

X-ray imaging projects X-ray photons towards the body, which is composed of different matter like bones and tissues. As the X-ray photons pass through the body, part of the energy is lost or scattered during collision with atoms which lie along the path. The amount of the X-ray energy remaining depends on the density and composition of the matter that collided with the X-ray photons [24]. The higher the density, the higher the mass attenuation coefficient, and thus more X-ray photons are attenuated. A detector placed behind the body then captures the remaining X-ray to form an image. The contrast in the image is due to the difference in the remaining X-ray between different locations.

In biomedical imaging, without the use of any externally administered CA, X-ray is commonly used to image the bones. This is because bones have a higher mass attenuation coefficient [24] as it is much denser compared to bodily fluids and soft tissues. Bones block more of the X-ray and this gives the contrast in the X-ray imaging of bones.

In order to image some other parts of the body, externally administered CA can be used. These include tri-iodobenzene, gold nanoparticles [27] and barium [18]. Barium has been used as an X-ray CA for many years. Due to its high atomic number and thus density, it is able to absorb X-ray effectively and often used to image the gastrointestinal tract. A 'barium meal' (barium sulphate in suspension) will be given to the patient prior X-ray imaging, and it coats the inner wall of the tract after oral administration. This CA is not targeted at any cancerous lesions and will line the entire tract. This makes the contour of the inner wall of the tract visible, so that any lesion big enough and on the surface can be detected. The barium meal does not cause adverse health effect to the patient, but it can still make one feels sick, such as constipation for a few days.

2.1.1.2 Single-photon emission computed tomography (SPECT)

SPECT uses radiotracers which are usually injected into the bloodstream of the patient. The radiotracers decay and directly produce one or more gamma photons. The gamma photons travel in random directions, and unlike visible light which is a low-energy photon, gamma photons cannot be focused by conventional lenses. Thus collimators are used so that the angle of the incoming gamma photons towards the detectors can be restricted. As a collimator instead of a focusing lens is used, the proportion of gamma photons that were emitted, directed towards and eventually measured by the detector is very low. Also, tissues are a good attenuator of the gamma photons released by SPECT isotopes. As the gamma photons are released within the tissues, they are mostly attenuated after travelling a short distance in the tissues before reaching the gamma detector. These two reasons coupled together make SPECT detects only an insignificant proportion of gamma photons produced at the lesions, thus making SPECT very insensitive [7].

Radiotracers are chosen and used based on its ability to attach itself to specific target structures like cancer tissues. Ideally, they should exhibit excellent tissue penetration, high affinity to target structure, specific uptake and retention only in the target cells [28]. They also have to be very stable *in vivo*, easy to prepare and non-toxic [28]. All these points are crucial so that the targeted cells can be easily detected and there is only minimal radiation risk to the patient.

2.1.1.3 Positron emission tomography (PET)

A positron is the anti-matter of electron having the same mass but opposite charge. Prior to imaging, positron emitting isotopes are introduced into the body which accumulates in the region of interest, acting like a tracer. The emitted positron will interact with an electron and undergoes annihilation, releasing a pair of gamma photons of the same energy (511 keV) but in opposite directions. Detection is done using a stationary ring sensor, which houses multiple pairs of highly sensitive detector, placed directly opposite each other. When a pair of detector detects a gamma photon within a “coincidence window” around 10 ns, it can be stated that the annihilation occurs along the line between the pair of detector [29].

SPECT and PET are similar to each other as both are able to detect small amounts of radioactive tracers. However, PET is 2-3 orders of magnitude more sensitive than SPECT and has better spatial resolution and quantification [28]. Compared to SPECT, one major drawback of PET is that the production of PET radioisotopes is more expensive and limited in variety [7,28]. This makes SPECT more frequently used for routine applications.

2.1.2 Medical imaging using non-ionising radiation

Contrast to ionising imaging, non-ionising imaging is free from all the inherent risks related to ionising radiation. Non-ionising radiation includes types of electromagnetic waves

which does not have sufficient energy to cause ionisation of tissues. This renders imaging methods using non-ionising radiation such as visible light, infrared and microwave radiation, safer and more suitable for regular check-up over a longer period of time.

2.1.2.1 Optical imaging

Optical imaging exploits ultraviolet, visible and near-infrared (NIR) light and can have a higher spatial resolution (about $0.1\ \mu\text{m}$ - $100\ \mu\text{m}$) compared to other common imaging techniques like magnetic resonance imaging ($10\ \mu\text{m}$ - $100\ \mu\text{m}$), X-ray imaging ($50\ \mu\text{m}$ - $200\ \mu\text{m}$), ultrasound imaging ($50\ \mu\text{m}$ - $500\ \mu\text{m}$) and PET ($1\ \text{mm}$ - $2\ \text{mm}$) [30]. Optical imaging is also able to detect cancer with lesser cancer cells per imaging voxel [7], making it more sensitive. This enables optical imaging to better detect small tumours that had just undergone subtle manifestation in the early stages of diseases.

Optical imaging is made up of many different groups of imaging, such as ballistic imaging, optical coherence tomography (OCT) and diffuse optical tomography. Each group is then further divided into different types, and some will be briefly discussed.

Ballistic imaging is based on unscattered or singly backscattered ballistic photons. However in many cases, quasi-ballistic photons are also measured to increase the otherwise very weak signal strength. Ballistic imaging gives high spatial resolution but very little penetration depth [31]. Some of the imaging types in this group are confocal microscopy and two-photon microscopy.

OCT offers a spatial resolution of $1\ \mu\text{m}$ - $10\ \mu\text{m}$ and the maximum penetration depth in biological tissues is $1\ \text{mm}$ - $2\ \text{mm}$. Consequently the depth-to-resolution ratio is more than 100. This makes OCT a high-resolution imaging. Contrast in OCT is mainly due to the

backscattering and the detection is based on interferometry [31]. Time-domain and Fourier-domain OCT are some of the imaging types in this group.

Though optical imaging can give high spatial resolution, one major drawback of optical imaging is its limited penetration depth. Light transfer is dominated by scattering in biological tissues. As light travels from the ballistic regime and into the diffusive regime, it undergoes much scattering. This transition takes place around one transport mean free path in biological tissues, of the order of 1 mm [31]. Beyond this depth, high-resolution optical imaging does not offer sufficient spatial resolution. This is due to light already undergoing significant scattering, deviating much from the original incidence direction, and thus making focusing ineffective. This maximum penetration depth of the order of 1 mm due to optical scattering represents its principle limitation.

One way to overcome this limitation is to use NIR. With lower absorption in tissues, NIR has a deeper penetration depth than visible light. The optical absorption of NIR by water and haemoglobin, which are abundant in tissues, is very little and thus able to have a penetration depth of several centimetres [32].

2.1.2.2 Ultrasound imaging (USI)

Ultrasound refers to sound waves above human audible range of 20 kHz. USI therefore has no risk of undesired radiation effects due to the use of ionising radiation. USI is based on the principle of pulse-echo imaging. The pulse in USI is produced by transducer using piezoelectric materials. Such materials convert mechanical energy to electricity, and *vice versa*. The material can either be a crystalline solid or ceramic. When a voltage having the same frequency as the resonance frequency of the piezoelectric material is applied to it, it

will in turn undergo vibration. These vibrations then produce vibrating pressure waves, and the pulse is transmitted to the environment as ultrasonic waves [33].

As the ultrasonic waves travels in biological sample, the density difference in biological tissues, fluids and bones, provide a mismatch in acoustic impedance which reflects ultrasound [34]. The echo (reflected ultrasound) travels back towards the piezoelectric material of the transducer, causing it to vibrate to produce a voltage when detected. The same transducer acts as both a transmitter and receiver of ultrasound.

The detected signals are analysed, and two important parameters can be extracted. First, the amplitude of the echo is a measure of the mismatch in acoustic impedance between the constituent materials within the sample. This gives the contrast in USI. The second parameter is the time of arrival of the echo. The longer the echo takes to reach the transducer, the further away from the transducer the echo was reflected. The echo's time of arrival can be easily converted to distance once the speed of sound of the bulk material is known. A spatial image which provides information on the density is then obtained.

However, the contrast of USI in early-stage cancer where lesion can be very small with only subtle physical manifestations may not be significant enough for it to be detected easily. USI can provide large penetration depth of up to 20 centimetres [35], due to its low ultrasonic scattering in tissue. Thus it can be used to give tissue structural information at deeper depth. It is also relatively small and portable can be used for bedside and clinical applications. USI is now commonly used in many clinical applications for diagnostic purposes in cardiology [36], gynaecology and obstetrics [37], as well as for therapeutic purposes in physical therapy and drug delivery [33].

2.1.2.3 Magnetic resonance imaging (MRI)

MRI makes use of a phenomenon known as magnetic resonance. It involves the atomic nucleus absorbing and re-emitting electromagnetic waves at a characteristic ‘resonant’ frequency (radio frequency range) under exposure to a strong magnetic field. MRI is considered safe for human as there is no known adverse effect from either the strong magnetic field or the radio wave [38].

Fig. 2.1: Precession as seen in (a) non-zero spin nuclei in external magnetic field and in (b) spinning top in gravitational field [38].

Magnetic resonance occurs due to some nuclei having tiny magnetic moments. When an external magnetic field is applied to a nucleus, the magnetic moment of the nucleus goes through a rotational motion called precession, instead of going into alignment with the magnetic field. This is similar to the slow wobbling motion in a spinning top (Fig. 2.1). The nucleus precesses about the magnetic field at a frequency known as Larmour frequency, which is proportional to the magnetic field strength and the nucleus’s gyromagnetic ratio. As the nucleus precesses about the magnetic field, it produces an oscillating magnetic field at the Larmour frequency. The net magnetic field oscillation from a sufficient number of nucleus precessing in a synchronised manner can be detected by a radio frequency receiver coil to produce the magnetic resonance signals [38].

When MRI is used for bio-applications, the most important nucleus is hydrogen as water and fats contain hydrogen and they can be found throughout the body. Hydrogen exhibit

magnetic resonance because it possesses net spin [38]. MRI is extremely versatile because of the wealth of information contained in the signals [38]. However, MRI systems have high operation and maintenance cost and patients with ferromagnetic implants are prohibited from using such systems due to the presence of the strong magnetic field.

Section B: Selected imaging modalities

2.2 Hyperspectral imaging (HSI)

HSI has been used in airborne and spaceborne remote sensing as early as 1989 [39], after the introduction of electronic recording system which replaced the film-based system [40]. This allows the intensity of narrow and adjacent spectral bands over a large spectral range to be recorded, giving rich spectral information in each spatial pixel. The detailed spectral signatures in each spatial pixel can be compared to the unique spectrum of known materials. This allows the classification and quantification of materials to those already in the data library, or to determine the presence of unknown materials. Not only has HSI been used for remote sensing [41], it has now been used in a wide variety of applications such as quality assessment of agriculture and farm products [42,43], biomedical applications [44] and forensics investigations [45,46].

HSI can be designed to provide high spectral resolution within its designed spectral range of interest, making it suitable for multiple fluorescence tags to be imaged simultaneously. The mixed emission of these tags can overlap spectrally and still be distinguished later during analysis. HSI overcomes the limitations of conventional spectroscopic imaging where the tags need to have minimal spectral overlap and each has a band-pass filter [47].

There is therefore no constraint in HSI on the number and combination of the tags that can be used in each imaging.

However, like any other optical imaging techniques, HSI will have an imaging depth of the order of 1 mm in biological tissues [31]. Beyond this depth, optical imaging does not offer good spatial resolution.

2.2.1 Classification of spectral imaging

Multispectral, hyperspectral (HS) and ultraspectral imaging are some of the common terms used in spectral imaging. In general, the differences between them in the above mentioned order are more number of wavelength bands and higher precision. However, there is no universally accepted guideline that differentiates one from the other. Two classification criteria are presented in Table 2.1. Both criteria use number of wavelength bands as one of the definition parameters. However, Fresse *et al.* [48] used precision while Puschell [49] used resolution. By using number of wavelength band, a common parameter between these two definitions, the latter makes it easier for one to classify a system as a HS or ultraspectral imager. The first definition is stricter, and is used to define the type of spectral imaging employed in this thesis. It should however not be used as a benchmark for other spectral imagers not presented in this thesis.

Table 2.1: Classification of spectral imaging.

Spectral imaging	Fresse <i>et al.</i> [48]		Puschell [49]	
	Number of wavelength band	Spectral precision ($\Delta\lambda/\lambda$)	Number of wavelength band	Spectral resolution (nm)
Multi	5-10	0.1	Order of 10	~20-100
Hyper	100-200	0.01	~30-300	< 10
Ultra	1000-10000	0.001	> 300	< 1

With more wavelength bands detected and an increase in spectral range, precision and resolution, HS and ultraspectral imaging can give more detailed spectral signatures that can be used for identification purposes with higher degree of accuracy. The high spectral resolution of ultraspectral imaging can be used to capture molecular absorption or emission band [50].

2.2.2 Datacube

HSI yields a datacube which is a three-dimensional (3-D) set of information in a spatial-spatial-spectral domain. Fig. 2.2 shows a cut-datacube where a portion of the datacube is removed to reveal its internal features. Multiple two-dimensional (2-D) spatial-spatial images each corresponding to a particular spectral band can be extracted from the datacube. Each voxel in the datacube holds the intensity-related information of a particular spectral band from one spatial point in the 2-D sample [51]. A spectrum is acquired by extracting the information from a spatial point along the spectral domain. Since the spectrum is made up of information from narrow and adjacent spectral bands, rich spectral information can be acquired. The spectrum can then be used for classification and quantification by comparing it with a set of data library using algorithms.

Fig. 2.2: 3-D cut-datacube [52].

Many methods are available to perform HSI, which can be divided into three main types, namely the spatial-scanning (whiskbroom and pushbroom), spectral-scanning and snapshot imagers. These methods differ in how the data are acquired to form the datacube (Fig. 2.3). Each method has its own advantages and limitations and should be chosen based on the requirements and applications.

Fig. 2.3: Data acquired in each scan by different HS imagers [53].

2.2.3 Major embodiments of table-top/field HSI

2.2.3.1 Spatial-scanning imager

Spatial-scanning HS imagers are commonly used in many table-top and field configurations [54-56]. It usually uses a dispersive element such as a prism-grating-prism assembly in a spectrograph [57] to split the incoming light so that the constituent wavelength bands can be detected by the sensor array of the camera. Spatial-scanning HS imagers can be further divided into two types, namely the whiskbroom and pushbroom imagers. Some of these systems have video cameras for direct video imaging [54,58].

A whiskbroom HS imager is point-scanning, and records the spectrum of only a spatial point in each scan to give one-dimensional (1-D) spectral information. By repeating the scan

across multiple points in a 2-D area, a datacube can be formed. Spatial scanning can be done using a 2-D stage to move the sample or using a micro-electro-mechanical system scanner to direct the point illumination to different parts of the sample [59]. With a large sample, the data acquisition time can be long as scanning needs to be repeated for each point in the sample.

Another method that can be used is the line-scanning pushbroom imager (Fig. 2.4) [47,60]. In each scan, a pushbroom imager is able to capture the spectrum from each point across a line of the sample. This is done by having a narrow slit which allows only a line of light to pass through [57]. The light from this line of the sample is dispersed into different wavelengths onto the 2-D sensor array. A 2-D spatial-spectral image is captured in each scan. Scanning is repeated after a relative displacement between the sample and the HS imager, in the direction transverse to the slit so that the next line can be imaged. Spatial-scanning can be done using a 1-D stage to move the sample, or by the linear displacement of the HS imager. After the entire region of interest (ROI) is imaged, the arrangement of the multiple 2-D data according to the sequence in which they were collected forms the datacube. Compared to point-scanning whiskbroom HS imager, the line-scanning pushbroom imager acquires more information in each scan. It is therefore a more efficient and faster alternative.

Fig. 2.4: Typical table-top pushbroom HS imager [61].

2.2.3.2 Spectral-scanning imager

Spectral-scanning HS imagers have been used in table-top and field configurations [51,62]. These systems use electronically tunable filters, such as acousto-optical tunable filter (AOTF) [63,64] and liquid crystal tunable filter (LCTF) [65,66]. AOTF (Fig. 2.5) and LCTF have spectral transmission that can be controlled electronically [51]. Each scan captured by the sensor is the image of the object at a particular spectral band, giving 2-D spatial-spatial information. By controlling the tunable filter to transmit different spectral bands, multiple spatial-spatial images are acquired to form the 3-D datacube. The time taken by an AOTF to switch from a wavelength band to another is below 1 ms [67], which is a few times faster than that of an LCTF. AOTF is thus preferred for video imaging application which requires higher frame rate [52].

Contrast to spatial-scanning imager, the number of wavelength bands to be recorded in spectral-scanning imager can be changed by user interference. This flexibility can reduce acquisition time when a lower number of wavelength band is required. Also, no relative motion between sample and detector is required between scans.

A fairer comparison in acquisition time between line-scanning and wavelength imager can be made with equal image size, number of wavelength band and exposure time. Acquisition time of a line-scanning imager is mainly affected by number of rows and the switching time between rows (varies by distance and motion speed). In the case of a wavelength imager, it is affected by the number of wavelength band and switching time between wavelengths.

When the sample is stationary, the data collected in both spatial and spectral-scanning imager will reflect the correct spectrum for each point in the sample. When a sample is moving in an unexpected manner, the data collected is distorted, but have different interpretation between these two imagers. If the sample moves unexpectedly only when row switching, the spectra recorded by the spatial-scanning imager is a right representation of specific points of a sample but placed in the wrong spatial position in the data. However, in spectral-scanning imager, such sample motion will result in incorrect spectra to be recorded for all points as wavelength information is taken sequentially. Therefore the requirement for a stationary sample during data acquisition is stricter in spectral-scanning imager recording large number of wavelength bands, though image registration algorithms can be used to correct the spectra [52].

Fig. 2.5: Schematic of AOTF [52].

2.2.3.3 Snapshot imager

Many table-top and field HSI systems are based on snapshot imagers [56,68,69]. Snapshot HSI systems are able to capture the 3-D data to build a datacube in a single scan [70]. This is done using different configurations such as integral field spectroscopy [71], image mapping spectroscopy [72], computed tomographic imaging spectroscopy [68] and compressive sensing [69]. Such systems do not need sequential scanning to build a datacube. The ability of the snapshot imager to capture the 3-D data in one scan has both advantages and limitations. The main benefit of such an imager is that it is much faster than spatial- and spectral-scanning HSI systems, and can be used in real-time applications depending on the exposure time and the detector's readout rate. Motion artifacts and pixel misregistration can therefore be eliminated [68]. Each 2-D detector has a limited number of pixels and can only capture that much information in one scan. Thus snapshot imager can only acquire a limited amount of information, and has to sacrifice on the number of spatial points or wavelengths from which the data are collected.

One of the configurations of a snapshot HSI system is integral field spectroscopy, which uses a reformatter that comes in different forms such as fiber bundle, box and rod (Fig. 2.6) [73,74]. The fiberlets on one end of the reformatter are arranged in a 2-D array, and the fiberlets on the other end are arranged in a 1-D row [71]. Light from the sample is captured by the fiberlets on the 2-D end of the reformatter, and through the fiberlets, the light is transferred to the 1-D end. The fiberlets on the 1-D end of the reformatter acts as a slit of a spectrograph-based HS imager. The use of such a reformatter in a HS imager allows the 2-D sensor array to capture 3-D spatial-spatial-spectral data (Fig. 2.7). Data processing is required to rearrange the acquired spectra according to the positions of the fiberlets on the

2-D end of the reformatter for the correct visualization of the data. In table-top and field configurations, integral field spectroscopy has been used in the field of astronomy and ocular imaging [75,76].

Fig. 2.6: Types of reformatter in integral field spectroscopy: (a) fiber bundle, (b) box and (c) rod [73,74].

Fig. 2.7: Integral field spectroscopy HS imager using fiber bundle reformatter [53].

2.2.4 Major embodiments of endoscopic HSI

Some diseases occur at sites within the body that are not easily accessible by conventional microscope setup. Endoscopes have been developed to satisfy the medical needs to image the human body from within. They can be used to detect cancers that occur in places which are not easily accessible using table-top systems, such as the gastrointestinal tract and oesophagus system. Although these sites are harder to access as they are located within the body, they are located near the hollow tracts in the body. Therefore flexible, thin

and small endoscopes can be used to access and image these sites. Endoscopes are also preferred to have fast image acquisition to guide the movement of the endoscope within the body. This can increase image quality by making the images less susceptible to motion artefacts due to natural movements of the muscles such as segmentation and peristalsis.

In recent years, a lot of effort and works have been carried out on hyperspectral endoscope, which uses spectral-scanning [65,67] and snapshot methods [21]. HS endoscope based on spatial-scanning method has not been reported. This may be due to spatial-scanning method being considered to be slow and thus not suitable for real-time application.

The probe of existing HS endoscopic systems use the common endoscopic setup where a fiber bundle is used to transfer the image of the sample from its distal end to the proximal end. Optical illumination can be delivered to the sample using the same fiber bundle or another light guide. HSI takes place on the proximal end of the probe where the HS imager is used to collect and detect the light exiting the proximal end. These HS endoscopes use a fiber bundle in its usual configuration [77] or a commercially-available endoscope [78] as customisation of the distal end of the fiber bundle is not required.

2.2.4.1 Spectral-scanning imager

Both AOTF and LCTF have been used in spectral-scanning HS endoscopes for biomedical applications, such as tissue classification and detection of cancer [52,65]. The tunable filter is positioned between the proximal end of the fiber bundle and the detector. Such imagers capture multiple 2-D spatial-spatial images one wavelength at a time to build a datacube. Therefore many spectral-scanning HS endoscopes capture data from a limited number of wavelengths to increase the rate at which the datacube is formed. Datacubes with 10-51 wavelengths have been acquired using spectral-scanning HS endoscopes [52,67].

2.2.4.2 Snapshot imager

In real-time endoscopic applications such as *in vivo* disease diagnosis and surgical monitoring, the snapshot imager is the preferred choice. Although many methods have been used in snapshot HS imagers in the table-top or field configurations, only the image mapping spectroscopy method has been used in a HS endoscope. It has an image mapper which plays a key role by spatially distributing light from neighbouring regions of the sample to isolated regions on the sensor array of the detector camera. A prism is in place to spectrally split the light into its constituent wavelengths before being detected by the camera (Fig. 2.8).

Fig. 2.8: Concept of image mapping spectroscopy [21].

Although HS endoscope using image mapping spectroscopy is fast, the assembly and alignment of such a system can be difficult. It requires the use of double Amici prism and lens array and the fabrication of the image mapper which involves machining (Fig. 2.9). Another drawback of this method is that only about 50 spectral bands can be acquired [21].

Fig. 2.9: HS endoscope using image mapping spectroscopy [21].

2.2.5 Contrast agents (CAs) used in HSI

Contrast in HSI is due to the unique optical spectrum of each component that is detected. In biomedical HSI, the reflection mode is commonly used as only one side of the imaged tissue is accessible by the imager in most cases. CA can be made to have specific binding to desired parts of a tissue. Thus these types of CA are applied so that they can be tagged to specific parts of the tissue to be imaged. Using HSI, multiple CAs can coexist on the same tissue and be detected at the same time, even though the spectra are closely overlapping.

2.2.5.1 Endogenous CAs

i. Tumours

HSI has been used to image tumours in both reflection and laser-induced fluorescence imaging modalities [62,78]. The spectra acquired from tumours and healthy tissues using

these two imaging modalities are distinct. One such experiment was carried out on mice injected with rat tracheal carcinoma cells [62]. Experiments using reflection imaging have also been used on pharynx (Fig. 2.10) and larynx tumours shortly after excision [52,63].

Fig. 2.10: (a) Expert labelling and (b) results of HSI after data analysis [63].

ii. Blood

Angiogenesis is a hallmark of cancer. It involves the process where neovasculature is formed for the tumours to supply them with nutrients and oxygen. At the same time, it removes the metabolic waste and facilitated the metastasis of tumours [79-81]. The higher density of blood vessels in tumour due to neovascularisation leads to a higher density of blood in tumour. Oxy-haemoglobin (HbO_2) and deoxy-haemoglobin (HbR) both exist at the same time in blood vessels to give total haemoglobin concentration (HbT).

The vasculature of the lower lip of human had been imaged in reflection imaging. The HSI system was able to differentiate between the vein and the surrounding tissues to give the vasculature patterns. The dominating feature in the reflection spectrum was attributed to the absorption peaks of oxy-haemoglobin [21]. HbO_2 and HbR exist in blood and their distinct spectra can be used to determine the blood oxygen saturation (sO_2) (Fig. 2.11) [68,82], an important hallmark of many diseases and cancers.

Fig. 2.11: (a) ROI and (b) blood sO_2 mapping of retinal vasculature [68].

iii. Lipids/carotenoids

Atherosclerosis is the formation of plaques in arteries, which is a slowly progressing condition leading to diseases such as heart attacks and strokes. Atherosclerotic plaques rich in lipids have a higher concentration of carotenoids, mainly beta-carotene, than normal aortic tissues. Beta-carotene has two distinctive absorption peaks at 450 nm and 480 nm, which can be used in HSI to detect its presence and serves as an indication of diseases (Fig. 2.12) [83].

Fig. 2.12: (a) ROI and (b) K-means classification overlays under white-light [83].

2.2.5.2 Exogenous CAs

i. Fluorescent microspheres

Different types of fluorescent microspheres have been used simultaneously for biomedical imaging applications [54,84]. Up to four types of fluorescent microspheres have

also been used at one time for the imaging of cells (Fig. 2.13). The fluorescent spectra of these microspheres are highly overlapping with the spectral emission peak occurring within about 50 nm. The acquired spectrum from each spatial pixel can be contributed by multiple types of fluorescent microspheres of varying concentrations. Using analysis algorithm such as multivariate curve resolution, the constituents spectra can be resolved to determine the relative concentrations of each fluorescent microsphere for each spatial pixel [58].

Fig. 2.13: ROI and acquired spectra from selected spatial pixels [54].

2.3 Photoacoustic imaging (PAI)

The photoacoustic (PA) effect was first report in 1880 by Alexander Graham Bell, but it was only until recently that more research on PA picked up. The introduction of computers, lasers and ultrasonic transducer eventually gave rise to PAI [31], which is a relatively new imaging modality that has been rapidly developing. It is a hybrid combination of rich optical contrast and high ultrasonic resolution, using optical excitation for ultrasonic detection. It uses safe non-ionising radiation and can have deeper imaging depth compared to many other types of pure optical imaging modalities. PA has been used in many applications such as biomedical imaging [85,86], chemical sensing [87] and the measurement of optical absorbance and Grüneisen parameter [88,89].

The main advantage of PAI is that it overcomes the penetration depth limit of the order of 1 mm in high-resolution optical imaging as ultrasonic scattering is much lesser than optical scattering in biological tissues [90]. PAI can give finer resolution at deeper penetration depth, up to a few centimetres [30,90,91], which remains a challenge for pure optical imaging which cannot go beyond the optical diffusion limit of the order of 1 mm.

2.3.1 Working principle

When pulsed optical excitation is irradiated onto tissue surface, part of the energy is absorbed by the tissue. The amount of energy absorbed is directly proportional to the local fluence and the wavelength-dependent optical absorption coefficient. The energy absorbed causes a transient temperature rise resulting in thermoelastic expansion which is dependent on the Grüneisen parameter. This results in the formation of initial pressure rise, producing broadband acoustic wave, also referred to as PA wave [85,92,93]. The PA waves can be detected by an ultrasonic transducer (UST) and the image contrast is based on the local fluence, optical absorption coefficient and Grüneisen parameter.

Depending on the relative position of the optical excitation on the sample and the UST, there are basically three main modes, namely the forward, backward and sideward (ring) mode. The forward (Fig. 2.14) and backward modes place the optical excitation on the opposite and same side as the UST, respectively. While the sideward mode places the UST perpendicular to the direction of the optical excitation. Both forward and backward modes work well when imaging objects closer to the UST, but at the same time imaging closer objects are more susceptible to noise. Since the sideward mode has a full view of the sample, images can be reconstructed with better precision [94].

Fig. 2.14: Forward mode PAI [95].

Each mode is suitable for different medical applications. The forward mode is easier to configure as the optical excitation and UST are on the opposite side. It can be used in cases where prototype concepts are to be tested. However, it may not be practical for use in an endoscope where the excitation and detection has to be on the same side. This is when the harder to configure backward mode is required. The sideward mode can be applied to image bulging body parts, such as breast imaging.

The image resolution and imaging depth in PAI is scalable with the ultrasonic frequency (function of laser pulse width, targeted imaging depth and frequency response of UST) and bandwidth, which when increased gives better spatial resolution at the expense of imaging depth [31]. Such scalability of PAI enables it to be used for many different applications by changing its design parameters.

2.3.2 Major embodiments of PAI

The scalability of PAI allows it to be configured to have different setups for the scaling of its spatial resolution and imaging depth. Currently, PAI comes in three major embodiments namely the focused-scanning PA microscopy, PA computed tomography and PA endoscopy.

2.3.2.1 PA microscopy (PAM)

PAM has focused optical excitation and ultrasonic detection where the dual foci are confocal to maximise the sensitivity of the system. Each scan provides an A-scan image which is 1-D in the depth-domain. Coupled with 1-D and 2-D spatial scanning will give 2-D depth-spatial and 3-D depth-spatial-spatial PA images, respectively [91]. PAM can be divided into 2 categories, optical-resolution PAM (OR-PAM) and acoustic-resolution PAM (AR-PAM), depending on whether the optical or ultrasonic focus gives better lateral resolution (Fig. 2.15).

Fig. 2.15: Configurations of (a) OR- and (b) AR-PAM [91].

OR-PAM provides high lateral resolution at cellular level about few hundred nanometres to a few micrometres. This is due to the use of the focused optical excitation by microscope objective to restrain PA excitation. OR-PAM can be used to image blood oxygen saturation in single capillaries without the use of exogenous CAs, with imaging depth within the optical diffusion limit of about 1.2 mm [96,97].

AR-PAM increases the imaging depth beyond the optical diffusion limit to about few millimetres. The high lateral resolution (tens of micrometres) in AR-PAM is due to the use of diffraction-limited acoustic detector. Lasers of higher power can be used for macroscopic

imaging to achieve imaging depths of centimetres. However, such lasers have low pulse rates and transverse scanning becomes too slow for many clinical applications [91].

2.3.2.2 PA computed tomography (PACT)

PACT has an UST array to increase data acquisition rate. The entire ROI is optically excited and the PA waves are simultaneously detected by the array of acoustic detectors. Inverse algorithm is used to reconstruct PA images by determining the locations of the sources of the PA waves from the acquired time-resolved PA signals [98]. Most UST arrays are 1-D and each scan gives a 2-D depth-spatial PA images. By moving the 1-D UST array in the direction orthogonal to the imaging plane, 3-D depth-spatial-spatial PA images can be acquired [91]. The 1-D UST array can be configured linearly or circularly, depending on the anatomy of the ROI (Fig. 2.16).

Fig. 2.16: Configurations of PACT using (a) linear- and (b) circular-array UST [91].

Linear-array PACT can only image the sample from one direction and has a partial-view detection where the detection angle by the linear-array UST of the ROI is less than 360° . In circular-array UST PACT, the ROI can be kept within the circular array. The PA waves from the ROI can be detected by the UST around all in-plane directions. This gives circular-array PACT full-view detection without missing boundary to provide PA images of higher quality compared to linear-array PACT.

2.3.2.3 PA endoscopy

PA endoscopy is used to image the internal body cavities from within the body by being able to bend around tight bends and corners to reach places which are difficult to access. One such PA endoscope has a side-fire optical excitation with internal scanning-motion mechanism (Fig. 2.17). It uses a rotating geared micro-motor and magnets in the PA endoscopic probe as a magnetic coupling mechanism to rotate the scanning mirror. Other components in the probe like the UST and optical fiber do not rotate.

Fig. 2.17: Side-fire scanning PA endoscope [99].

The optical fiber goes through the central hole of the single-element UST. The light emerging from the end-face of the fiber serves as optical excitation and is directed by the scanning mirror. The reflective surface of the scanning mirror is 45° to the optical axis of the optical fiber, thus the light is perpendicular to the optical fiber and catheter [99]. As the scanning mirror rotates, light from the optical fiber is reflected sideward to different points on the tissue. The scanning mirror is also used to direct the PA waves from the tissue and the UST.

Another PA endoscope adopts a snapshot design which does not require any motor to rotate the PA endoscopic probe or parts of it (Fig. 2.18). The optical fiber passes through the hole of a circular-array UST. The optical illumination exiting the end-face of the fiber is reflected by a taper reflector [100] located at the terminal end of the optical fiber. This

enables the light to be reflected in a ring beam. When placed in a hollow tissue, light exiting the endoscopic probe forms a ring illumination on the tissue surface.

Fig. 2.18: Snapshot PA endoscope [100].

The PA waves generated by the tissue are redirected by the taper reflector and onto the 64-element circular-array UST. This allows detection of PA waves from all directions at once. A single laser pulse is able to capture a full ring-view PA image of the object due to parallel acquisition of the 64-element circular-array UST. This eliminates the need for the mechanical rotation of the PA endoscopic probe. Compared to side-fire scanning PA endoscope, the snapshot design is better suited for high-speed applications (no scanning) and relatively simpler to assemble as it does not require any rotating mechanism. It does however require the addition of a taper reflector and multi-element circular-array UST.

2.3.3 Theory

Two important timescales exist in laser heating for PAI, which are the thermal relaxation time and stress relaxation time. If the laser pulse width is much shorter than the thermal relaxation time, the excitation is considered to be in thermal confinement and heat conduction is insignificant during the laser excitation. Similarly, if the laser pulse width is much shorter than the stress relaxation time, the excitation is considered to be in stress confinement and the stress propagation is insignificant during the laser excitation [31].

By meeting these two conditions, PA phenomenon occurs and the generation and propagation of PA waves in an acoustically homogenous and nonviscid infinite medium can be described as shown below [101,102],

$$\left(\nabla^2 - \frac{1}{v_s^2} \frac{\partial^2}{\partial t^2}\right) P(\mathbf{r}, t) = -\frac{\beta}{C_p} \frac{\partial}{\partial t} H(\mathbf{r}, t), \quad (2.1)$$

where $P(\mathbf{r}, t)$ is the acoustic pressure at position $\mathbf{r}$ and time t , v_s is the speed of sound in the medium, β is the thermal coefficient of volume expansion, C_p is the isobaric specific heat capacity and $H(\mathbf{r}, t)$ is the heating function defined as the thermal energy converted at $\mathbf{r}$ and t per unit volume and time. For optical absorption, the heating function

$$H(\mathbf{r}, t) = \eta_{th} \mu \Phi(\mathbf{r}, t), \quad (2.2)$$

where η_{th} is the percentage energy converted into heat, μ is the optical absorption coefficient and Φ is the optical fluence rate [101].

In general, the initial pressure rise of the PA wave P_0 at $\mathbf{r}$ immediately after excitation by optical laser pulse is shown below [103]:

$$P_0(\mathbf{r}) = \eta_{th} \Gamma(\mathbf{r}) F(\mathbf{r}) \mu(\mathbf{r}), \quad (2.3)$$

where $\Gamma = (\beta v_s^2)/C_p$ is the dimensionless Grüneisen parameter and F is the optical fluence. In many cases, η_{th} is approximately equal to 1 [101]. Γ is temperature-dependent and both μ and F are wavelength-dependent. Without considering position $\mathbf{r}$, Eq. (2.3) can be written as shown below [31,93,104]:

$$P_0(\text{Temp}, \lambda) = \Gamma(\text{Temp}) F(\lambda) \mu(\lambda), \quad (2.4)$$

where Temp is the temperature of the medium and λ is the optical excitation wavelength.

It is important to note from Eq. (2.4) that the amplitude of the PA wave is directly proportional to Γ , F and μ . When comparing the properties of multiple samples based on the amplitude of the PA wave under the same experimental conditions (constant Temp, λ and F), both Γ and μ have to be considered. A highly-absorbing (high μ) sample can produce a weak PA wave if its Γ is very low. The effect of Γ on the amplitude of the PA wave should not be neglected.

Γ of a material describes how a change in temperature affects the size of the structure. It is independent of the optical excitation wavelength, but dependent on the temperature as well as the physical properties of the sample. Γ changes only slightly in water-based tissues kept at a constant temperature [104]. It is calculated to have an approximate value of 0.20 at the body temperature of 37 °C [31]. It is thus often regarded as a constant in water-based tissues when temperature is kept constant. Equation (2.4) is then expressed as

$$P_0(\lambda) \propto F(\lambda)\mu(\lambda). \quad (2.5)$$

In many pulsed laser system, the fluence of each pulse of the same wavelength can vary slightly, but it can vary more when the optical wavelength changes. When only a wavelength is used, a photodiode can be used to determine the pulse-to-pulse energy variations and account for such variations in the experimental results.

PA setups using few tens of wavelength bands have been reported [105,106]. In some of these cases, a photodiode with known responsivity can be used to measure the fluence on the photodiode. However, the ratio of the fluence of different wavelengths reaching the photodiode may not be the same as that reaching the tissue. The laser is usually split into two beams by an optical element such as a beam sampler, one of which is directed towards the photodiode and the other towards the tissue. The laser along these two paths may pass through different optical components which can be wavelength-dependent. Therefore, even if the photodiode is used to account for wavelength-dependent fluence fluctuations, it may not be measuring the actual fluence ratio on the tissue correctly. This can be solved by performing spectral calibration after all optical components are in place, or to assume that the optical properties of the optical components along the two light paths are independent of wavelengths.

When the laser pulse hits and travels into the tissue, fluence is reduced as light encounters optical scattering and absorption. Unless the optical scattering and absorption properties of the tissue at each position are known, it can be difficult to account for such fluence variations with position. This is especially so in heterogeneous tissue which is made up of different parts of unknown properties.

2.3.4 Point-illumination PAI using single-element unfocused UST

Although μ can be calculated using Eq. (2.4), it is not usually done so. From the experimental point of view, it is cumbersome to determine the actual values of P_0 , Γ and F to calculate the actual value of μ . In many cases, P_0 and F are measured in arbitrary values and Γ is considered to be a constant for water-based tissue [31].

The measure of the strength of P_0 is acquired from the signals of the UST ($P_{UST,raw}$) in a form of voltage against time t . Hilbert transformation is commonly used to process such analytical signals as it can be used to pick up the envelopes of vibration signals [107].

$$P_{UST}(\lambda, t) = \text{Hilbert}[P_{UST,raw}(\lambda, t)]. \quad (2.6)$$

The measure of the strength of F is acquired from the signals of the photodiode ($F_{PD,raw}$). The photodiode's responsivity Resp has to be taken into account for an accurate measurement of the fluence ratio of multiple wavelengths.

$$F_{PD}(\lambda) = \frac{F_{PD,raw}(\lambda)}{\text{Resp}(\lambda)}. \quad (2.7)$$

By making μ the subject and in terms of experimental data, Eq. (2.5) becomes

$$\mu(\lambda, t) \propto \frac{P_{UST}(\lambda, t)}{F_{PD}(\lambda)}. \quad (2.8)$$

Using an unfocused transducer, t can be converted into distance along the direction of detection of the UST (z) by assuming a fixed speed of sound in the tissue. This gives μ for each point along the z -direction for each signal. By putting together multiple signals across

the scanned direction (x), an x - z spatial mapping of μ can be acquired for each wavelength to show the PA image of the tissue, as shown in Eq. (2.9). Since variations of F are accounted for and Γ is considered to be a constant, the PA image will reveal position within the tissue where μ is high. In the case of a tissue, this can be used to locate tumours, as they are known to have higher optical absorption [100].

$$\mu(x, z) \propto \frac{P_{UST}(x, z)}{F_{PD}(x)}. \quad (2.9)$$

When only one wavelength is used during PAI, $Resp$ becomes a constant as it is a function of wavelength. Signals from the photodiode can still be acquired to account for the single-wavelength pulse-to-pulse fluence fluctuations, using Eq. (2.10). If it is assumed that there is no pulse-to-pulse fluence fluctuation, Eq. (2.10) can be further simplified to Eq. (2.11). Therefore, PA images show μ when Γ is considered to be a constant in water-based tissues and when fluctuations in F is accounted for or considered to be negligible.

$$\mu(x, z) \propto \frac{P_{UST}(x, z)}{F_{PD, raw}(x)}. \quad (2.10)$$

$$\mu(x, z) \propto P_{UST}(x, z) \quad (2.11)$$

In this case, the selection of wavelength becomes very important as it affects the quality of the PA image. μ is inherently a function of the optical excitation wavelength. There are wavelengths where μ of a tissue is high but also others where μ is low. The wavelength should be selected such that μ of the selected wavelength of the target such as vasculature and melanin is different than that of the ambient tissue surroundings [35,92]. This will give a higher contrast for a PA image with better quality. If the wavelength is selected such that μ of the target is very close to that of the surrounding, then the image will be relatively flat and the target will not be seen in the PA image clearly.

2.3.5 Contrast agents (CAs) used in PAI

Contrast in PAI is due to the presence of tissues with different optical absorption properties at some excitation wavelengths. Each CA can provide information on a certain aspect of the tissue. When more than one CA is detected, PAI can provide more complementary information on the tissue being illuminated. These include the depth, size, type and concentration of each CA, and from here other functional information can be known. Multiple CAs can coexist in the same tissue and can also be detected at the same time using multiple excitation wavelengths [85,108-113]. The number of excitation wavelengths used must be equal to or greater than the number of CAs to be identified. Post-processing, such as linear least squares unmixing [85,110], can be carried out to determine the abundance of each CA present. A mixture of endogenous and exogenous CAs can also be imaged at the same time.

2.3.5.1 Endogenous CAs

Endogenous CA is naturally occurring within certain sites in the body. Therefore they do not need to be artificially introduced to the ROI. This reduces the risk due to the presence of foreign materials in the body. The presence of these CAs in particular regions in the body indicates certain hallmarks of potential diseases.

i. Tumours

Human cancer tissue has been successfully detected *ex vivo* using a snapshot PA endoscope (Fig. 2.19). The cancer tissue can be detected as it produces stronger PA signals than the surrounding healthy tissue. This is due to the cancer tissue having a higher optical absorption at wavelength of 1064 nm [100].

Fig. 2.19: PAI of colorectal cancer tissue [100].

ii. Blood

Vasculature mapping and tumour detection due to its higher blood density are supported when using an optical wavelength where blood has significantly higher absorption than surrounding healthy tissues (Fig. 2.20). An accurate measurement of HbT using a single wavelength can be acquired using only the isobestic wavelength. HbT is then calculated using the same reference absorbance between HbO₂ and HbR. When more than one wavelength are allowed, abundance of HbO₂ and HbR can both be determined using information of their known absorbance values at the wavelengths used. HbT and blood sO₂ can also be subsequently calculated [85,110]. With blood sO₂ known, hypoxia or hypermetabolism, another condition which is very common in tumour [22,110] can thus be evaluated. It is the state where tissues do not have sufficient oxygen supply. This is caused by the uneven distribution of blood vessels in tumours, causing some regions to be low in oxygen supply, resulting in a lower blood sO₂ [30]. The distributions of HbT and blood sO₂ of the inside of rabbit oesophagus using PAI are shown in Fig. 2.20, giving critical structural, functional and physiological information on tumour growth and condition.

Fig. 2.20: PAI showing distributions of (a) HbT and (b) blood sO_2 [109].

iii. Lipid

PA intravascular imaging of the vascular tissue detects tissues rich in lipid, which is a sign of potential rupture risk leading to acute coronary events [114,115]. Lipids in atherosclerotic plaques in the presence of luminal blood have been detected when tested using animal (Fig. 2.21) or human arteries.

Fig. 2.21: PAI of lipids [114].

iv. Melanin

Although melanoma is the deadliest form of skin cancer, the prognosis can be good with early detection and treatment. Conventional diagnosis of melanoma is inaccurate and invasive, due to visual inspection and biopsy. PAI can be used as an accurate and non-invasive method to diagnose melanoma by determining the concentration of melanin [22]. The imaging of the anatomy of melanoma and the surrounding vasculature can be used to understand the growth and staging of such tumours (Fig. 2.22) [92].

Fig. 2.22: PAI of melanin [92].

2.3.5.2 Exogenous CAs

Exogenous CAs can be intravenously administered into the bloodstream or applied onto tissue surface to increase the contrast of specific targets in PAI. They bind themselves to specific targets, like lymphatic system and macrophages, and have higher optical absorption in relation to the tissues in the surrounding. Some exogenous CAs still need to go through further studies to ensure that they are stable and safe to be used on humans without undesirable effects [22,30,116].

i. Gold nanoparticles (NPs)

The use of NP as CA in biomedical imaging has recently garnered a lot of attention [117]. Gold NP undergoes surface plasmon resonance [118], where light scattering is

largely due to the collective oscillation of conduction electrons induced by light [119]. It is therefore a plasmonic NP. Gold NP is optically tunable over a broad spectrum from the near-ultraviolet to mid-infrared [120,121], by ways such as altering its shape (aspect ratio) [122] and the relative dimensions of the core and shells [120,121]. When tuned to the NIR region where tissue transmissivity is high, the imaging of thick tissues is allowed due to the deep penetration of light. Gold NP has a surface coating of gold which is biocompatible, making it a suitable CA for bio-imaging [118,121].

Gold NP offers a lot of advantages which should be exploited for biomedical imaging. However, another important factor which also needs to be considered is the cytotoxicity of using such CAs. It has been reported that there is no or insignificant adverse effects to cells when a low dosage of gold nanoshell is used [116]. There should be a balance in which these CAs are used effectively and safely for biomedical applications.

NPs are internalised by macrophages, one of the critical components of coronary heart diseases, and are aggregated within the cells. Macrophages loaded with gold NPs in a diseased rabbit aorta have been detected by PAI, and have shown to produce stronger PA signals at the injection sites denoted by the green arrows in Fig. 2.23 [108].

Fig. 2.23: PAI of macrophages loaded with gold NP [108].

ii. Organic dyes: Evans blue dye and potential other dyes

The lymphatic system is usually not detectable and imaged during PAI due to its low optical absorption. Therefore to image the lymphatic system, the Evans blue dye which acts as a CA is used. After the injection of Evans blue dye into rats, its lymphatic nodes and vessels near the colon have been imaged by PAI (Fig. 2.24) [109]. Other organic dyes have also been successfully used in PAI, which include the use of IRDye800-2DG to measure tumour glucose metabolism [85].

Fig. 2.24: PA image of Evans blue dye, supplementary notes of [109].

iii. Fluorescent probes

Alexa Fluor 750, a common NIR fluorescent dye has been injected below the knee joint of a euthanised mice for PAI. Although the targeted imaging plane is a challenging one due to the presence of tissues which are optically heterogeneous and bones of acoustically mismatched impedance, the dye can still be imaged using multispectral fitting (Fig. 2.25) [123]. Fluorescent proteins such as mCherry and eGFP have also been used for the PAI of the vertebral column of an adult zebrafish [112].

Fig. 2.25: PA image indicating the location of injected fluorescent dye [123].

Section C: Outcome of literature review

2.4 Overview of imaging modalities mentioned

Table 2.2 and Table 2.3 compare the ionising and non-ionising imaging modalities. Common parameters such as lateral resolution and imaging depth are included. The two targeted diseases in this thesis are colon cancer and uveal melanoma in the iris. In order to detect colon cancer in the early stages, high spatial resolution of the order of 100 μm and spectral resolution of about 1 nm are required to detect the subtle changes on the surface of tissues. For the detection of uveal melanoma in the iris, an imaging modality with a spatial resolution of the order of 1 mm and capable of producing different responses to different excitation wavelengths is required. The use of CAs with specific bindings to uveal melanoma is also preferred to produce images with enhanced contrast. The availability of structural information of the eye can also help to pinpoint the location of diseased sites.

Table 2.2: Summary of ionising biomedical imaging modalities.

Modality	X-ray	SPECT	PET
Endogenous CA	Bone	Not applicable	Not applicable
Contrast	Density	Radiotracer emitting gamma	Radiotracer emitting positron
Lateral resolution [30]	50 μm - 200 μm	1 mm - 2 mm	1 mm - 2 mm
Imaging depth	Deep (whole body)	Deep (whole body)	Deep (whole body)
Available probe/endoscope design	No	No	No
Notes	Requires exogenous CA such as barium to image soft tissues like colon	Bulky, insensitive, use of exogenous radiotracer	Bulky, insensitive, use of exogenous radiotracer which is expensive and of limited variety

Table 2.3: Summary of non-ionising biomedical imaging modalities.

Modality	Optical (microscopic)	USI	MRI	PAI	HSI (microscopic)
Endogenous CA	Blood, melanin	Bone, muscles	Fat, fluid	Blood, lipid, melanin	Blood, lipid, melanin
Contrast	Absorption, reflection, transmission, fluorescence	Acoustic impedance	Nucleus with net spin (hydrogen)	Optical absorption coefficient	Absorption, reflection, transmission, fluorescence
Lateral resolution [30]	~0.1 μm - 100 μm	50 μm - 500 μm	10 μm - 100 μm	220 nm - 720 μm [90]	~0.1 μm - 100 μm
Imaging depth	Orders of 1 mm [31]	Deep (foetus)	Deep (whole body)	100 μm - 7 cm [90]	Orders of 1 mm [31]
Available probe/ endoscope design	Yes	Yes	Yes	Yes	Yes
Notes	Many different types of configurations for a variety of applications	Inexpensive, portable, quick	Usually bulky and expensive	Very wide range of lateral resolution and depth by changing setup and optical or acoustic parameters	Compared to other optical imaging, has rich spectral information

Imaging modalities in Table 2.2 use ionising radiation. They do not encourage regular and frequent checks due to radiation risks. Furthermore, SPECT and PET require exogenous CAs to be introduced into the body. X-ray does not necessary need exogenous CA for bone imaging. However, when imaging the colon or other soft tissues, exogenous CA like barium is required. Generally, though they have very deep imaging depth, their lateral resolutions are not as superior to many of those in non-ionising imaging modalities. The lack of probe-based and endoscopic designs for these modalities also makes it very challenging to

implement them in endoscopes and probes for imaging the colon and eye. All these factors thus make imaging using ionising radiation unsuitable for the targeted research objectives from the perspective of the targeted diseases.

On the other hand, imaging modalities in Table 2.3 are non-ionising. Therefore they are free from all the radiation risks which are faced by ionising imaging modalities. The non-ionising modalities here also do not need any exogenous CA, though they can be used to further enhance the contrast in the image. In this group, USI and MRI have relatively lower lateral resolution and this makes them not so suitable for high-resolution imaging.

2.4.1 Endoscopic HSI for colon imaging

A common method to detect early colon cancer is to use white light colonoscopy [18]. An endoscope is used to image the colorectal region directly, and a clinician tries to identify the lesions in the image. Lesions that are flat, depressed and subtle present in the image may not be recognised by the clinician, as they are not easily identifiable [19]. This also depends on the clinician's experience and expertise. A way to reduce the variations among clinicians' performance is to use chromo-endoscopy (dye spraying), but it is not proven to better colonoscopy done by high-performance clinicians [19]. Detecting lesions using colonoscopy and similar methods will to a certain extent be affected by error in human judgement, especially for small lesions with subtle changes.

HSI can be superior to optical imaging of similar configurations as HSI records the intensity of narrow and adjacent spectral bands over large spectral range. This gives the spectral signatures to create a data library which can be used for classification and quantification in computer-aided diagnosis. HSI can be used to help clinicians make better diagnostic evaluation and confirmation of diseases. This removes the need for the actual

tissue excision as the results can be known on the spot. The availability of HS endoscopes also makes HSI suitable for colon imaging for the detection of diseases. These factors together make HSI a potentially very useful imaging modality in the detection and diagnosis of colon cancer. A comparison between white light colonoscopy, chromo-endoscopy and HSI for colon cancer detection is seen in Table 2.4.

Table 2.4: Comparison between conventional optical imaging methods and HSI for colon cancer detection.

Modality	White light colonoscopy	Chromo-endoscopy (dye spraying)	HSI
Use of dye/stain	No	Yes	No
Spectral range	Narrow (visible)	Narrow (visible)	Broad (visible to NIR)
Creation of detailed spectral data library	No	No	Yes
Computer-aided diagnosis based on spectral information	No	No	Yes

HSI is suitable for tumour detection in the colon using both reflection and fluorescence imaging modalities as normal tissue and tumour have different optical properties. The possible mechanisms resulting in the optical reflectance between normal tissue and tumour to be different include mucosal thickening and higher vasculature density in abnormal lesions [20]. The differences can be more easily detected when the tumour is located on the colon surface.

Endogenous fluorophores such as flavin, collagen and porphyrins [20,124,125] are natural fluorophores. Autofluorescence occurs when these endogenous fluorophores are excited by a laser having an appropriate excitation wavelength. The differences in the tissue microarchitecture and concentration of endogenous fluorophore between a normal tissue

and tumour [20] can lead to variations in autofluorescence [20,124,126]. Depending on the excitation wavelength, autofluorescence in tumour can be lower [20,126] or higher [126] compared to normal tissues. This is due to the excited endogenous fluorophores having lower or higher concentrations in tumour, respectively [126]. The magnitude of autofluorescence is dependent on the tumour stage since the differences between a normal tissue and tumour become greater as the tumour progresses. Therefore, detection and staging of colon tumour can be done using fluorescence imaging to capture the autofluorescence in tissues by looking out for changes in the fluorescence intensity.

Based on literature review, many spatial-scanning HS imagers do not come with a video camera. While those with video cameras are used for direct video imaging, there is no spatial synchronisation between the detector and video cameras, which could have been used to create a user-selectable ROI. This can help to minimise the data acquisition time, size of data and computation time. Even though HS endoscopes have been reported, their configurations are limited to only spectral-scanning and snapshot imagers. HS endoscope using spatial-scanning method has not been reported. Also, the reported snapshot HS endoscopes can only capture about 50 wavelengths [21].

2.4.2 PAI for ocular imaging

Uveal melanoma can be detected using a few imaging methods, such as ophthalmoscopy, fluorescein angiography and ultrasonography [15]. Although these methods are useful, it can only provide limited information when used on its own, which may not be sufficient for the diagnosis of uveal melanoma. Ophthalmoscopy is used for the diagnosis of posterior ocular tumours and thus not suitable to detect uveal melanoma in the iris [15]. Fluorescein angiography requires the introduction of dyes, which as fluorescein and indocyanine green,

into the body. Although it defines the tumour margin, the depth of the tumour cannot be determined to give the volumetric size of the tumour [15]. Ultrasonography is useful for measuring tumour dimensions [15], which gives only structural information based on density differences. These diagnosis methods would only be able to provide limited structural information without the capability to acquire information for disease staging.

The use of PAI offers many advantages in biomedical imaging. Firstly, PAI has a rich variety of optical contrast, both endogenous and exogenous. In tissues, naturally occurring CAs like HbO₂ and HbR are present. Therefore, PAI can be applied to many regions in the body such as the eye. Secondly, PAI is multi-scale and therefore flexible. It can increase lateral resolution at the expense of imaging depth, enabling it to have different configurations for varying applications and requirements. It has a very wide range of lateral resolution and imaging depth due to this factor. Also, multiple wavelengths can be used in PAI so that healthy tissues and tumours with different optical absorption can be spectrally separated [85]. These factors give PAI the potential to being a very suitable imaging modality in the diagnosis of uveal melanoma.

Based on literature review, PA methods have been used to measure the absorption-related properties of bio-samples with at most few tens of wavelength bands [105,106]. Photodiodes are sometimes used to measure the fluence reaching the sample without taking into account the wavelength-dependent optical characteristics of the optical components between the photodiode and the sample. This assumption may not always be true and the fluence ratio measured by the photodiode may not necessarily be the fluence ratio on the sample. Also, a snapshot PA imager for ocular imaging for fast data acquisition and real-time applications has not been reported.

2.4.2.1 Hybrid-modality imaging

Modern medical imaging modalities are efficient enough to provide the comprehensive structural, functional, and molecular information that will enable highly accurate disease diagnosis. Each imaging modality when use on its own, has its own advantages and limitations. However, the use of each imaging modality in a specific configuration is only suitable for certain diagnostic applications. By restricting to using just one modality, it is possible that there are many scenarios where the information provided may not be sufficient for a good diagnostic evaluation and confirmation of uveal melanoma.

Multimodality imaging is the use of more than one imaging modalities integrated in a single setting to acquire more information. The modalities chosen for integration should provide complementary and useful information for diagnostic applications. Using this approach, the benefits of each modality can be used to overcome the limitations of the other and to provide more information than could have been provided by only one imaging modality [23]. It can be used to give more useful structural, functional and molecular information compared to just using one modality [23]. This can also contribute to guided biopsy for higher accuracy, or may even lead to optical biopsy without the need to perform invasive tissue biopsy or needle aspiration which can be harmful to the patient.

In addition, multimodality imaging also helps to reduce the patient's level of discomfort when different imaging modalities have to be used. Instead of going through several screenings for different imaging modalities, a multimodality imaging system which has been integrated into a single setting will help to reduce patients' stress. Both the clinician and patient would benefit from the reduced screening duration. Thus there is a strong need to combine more than one imaging modalities.

In this thesis, the term hybrid-modality imaging refers to a subset of multimodality imaging which employs the use of imaging modalities that have different operation principles. For example, a multimodality imaging using reflectance and fluorescence imaging will not be considered as a hybrid-modality imaging, as these two modalities are both optical in nature.

It is important to note that PAI is commonly integrated with USI as both imaging modalities are detecting acoustic waves using an UST. USI has already been widely used and accepted in many clinical applications. By combining these two imaging modalities, it also makes it easier for clinicians to accept PAI as an emerging imaging modality [22]. By combining PAI and USI for ocular imaging, the optical absorption-based information can be made available through PAI and structural information is acquired through USI. Such integration can reveal the location of the tumour with respect to other ocular structures.

Together with the advantages of using PAI mentioned in Sec. 2.4.2, these factors make PAI a potentially very useful imaging modality in the detection of uveal melanoma in the iris (Table 2.5). A comparison between fluorescein angiography, ultrasonography and hybrid-modality imaging (PAI and USI) for uveal melanoma detection in the iris is seen in Table 2.5.

Table 2.5: Comparison between conventional imaging methods and hybrid-modality imaging for uveal melanoma detection.

Modality	Fluorescein angiography	Ultrasonography	Hybrid-modality (PAI and USI)
Use of dye/stain	Yes	No	No
Provides structural information	Yes (tumour margin)	Yes (tumour dimensions based on density differences)	Yes (tumour dimensions based on density differences)
Spectral range	Narrow (visible)	Not applicable	Broad (visible to NIR)
Creation of detailed spectral data library	No	No	Yes
Computer-aided diagnosis based on spectral information	No	No	Yes

The next chapter discusses the custom-designed and in-house developed pushbroom HSI system with a video camera incorporated to enable direct video imaging and for the selection of the ROI within its field of view. The benefits of having such features and the methodology and calibrations of the system will be discussed.

Chapter 3: Pushbroom hyperspectral imaging system with selectable region of interest

This chapter presents a spatial-scanning pushbroom hyperspectral imaging system incorporating a video camera, which is not only used for direct video imaging but also for the selection of the region of interest within the field of view of the video camera. Using a video camera for these two applications brings many benefits to a pushbroom hyperspectral imaging system, such as a minimal data acquisition time and smaller data storage requirement. A detailed description of the system followed by the methods and formulas used for calibration and electronic hardware interfacing are discussed. The experimental results are analysed using United States Air Force resolution chart, chicken breast tissue, and fluorescent targets as test samples.

3.1 Introduction

A few spatial-scanning hyperspectral imaging (HSI) systems have been reported in literature for biomedical-related applications. Some of these hyperspectral (HS) imagers do not use a video camera in the system [47,127], whereas others incorporate a video camera in the setup for direct video imaging [54,58], which has many benefits. Using a video camera for direct video imaging gives a better visual representation by providing colour images, which can be used to verify the data after measurement. The detector and video cameras can be positioned such that both cameras capture a focused image simultaneously. Using the video camera, samples of different thicknesses can be easily positioned to maintain the same working distance. It allows the sample to be positioned precisely and this is especially important for a system with small field of view (FOV). Unwanted and repeated scanning can be prevented to save time and minimise deterioration of the sample. However, having only direct video imaging capability does not allow the user to pinpoint exactly which area in the video camera's FOV to be the region of interest (ROI).

In this context, this chapter details the instrumentation, calibration, and the theoretical framework used to set up a pushbroom HS imager incorporating a video camera for both direct video imaging and user-selectable ROI. The advantages of using such a configuration include the benefits for direct video imaging mentioned earlier. The function introduced for user-selectable ROI allows the storing of the information from only within the ROI, minimizing measurement time, data size, and computational time. This precise mining of information from only within the ROI is accomplished by mechanical and digital means. While the top-to-bottom scanning of the ROI (height) is done by an automated motorised scanning stage, the mining of data from only the spectral range of interest and within the width of the ROI is done by digital means.

3.2 Instrumentation of pushbroom HSI system

The proposed pushbroom HS imager's design and configuration are shown in Fig. 3.1. The choice of the key components was affected by a few factors. The configuration of the system was first determined to have a quadrocular adapter (Y-QT, Nikon) to attach the spectrograph and detector and video cameras. The spectral range of interest was to cover the visible to near-infrared wavelength band, and thus the spectrograph with a spectral range from 400 nm - 1000 nm was chosen. It should also have a low keystone and smile distortions for better data quality. Therefore the spectrograph V10E ImSpectors from Specim (dispersion 97.5 nm/mm, numerical aperture F/2.4, slit width 18 μ m) was chosen over others in the same series. The detector camera should also have a spectral range similar to that of the spectrograph, so that the overall spectral range of the system is not reduced. It should also have a small pixel size for fine resolution and good sensitivity so that weak signals can be detected. The camera LucaEM DL-604M-OEM from Andor was selected to

be the detector camera. It has a spectral range of 400 nm - 1000 nm and pixel size of $8 \times 8 \mu\text{m}^2$ and uses electron multiplying charge coupled device technology for detection of weak signals. The forelens along with the focus adapter (2-16265, Navitar) were used for fine focusing. The doublet lens 2-50145 from Navitar (focal length: 95.2 mm) was chosen as the forelens so that the image size of the video camera (UI-1550LE-C-HQ, iDS) is of the order of a few millimetres. Further, this ensures a good working distance of more than 20 cm for the user to keep the sample as well as to enable the relevant opto-mechanical alignment. The three-axis motorised stage uses Physik Instrumente's compact micro-translation stages M-112.2DG in the x and y axes and M-110.1DG for the z axis. The stages have a minimum incremental motion of $0.2 \mu\text{m}$ which were found to be sufficiently good for the system. The stages in the x and y axes have a longer travel range of 25 mm for lateral positioning of sample, while the stage in the z axis has a travel range of 5 mm for axial positioning of sample.

Fig. 3.1: Schematic diagram of pushbroom HSI system.

The three-axis motorised stage is used to position the sample prior HSI. The y -axis stage is used to move the sample between each scan. Light from the sample passed through the doublet forelens which is placed in a fine focus adapter. This adapter is attached to the bottom side of the quadrocular adapter, which houses a sliding mirror. The sliding mirror is

initially pushed into the quadrocular adapter and directed light toward the video camera (Path 1 in Fig. 3.1) before scanning commenced. The video camera allows direct video imaging of the sample. The software developed allows the user to choose a particular region within this FOV as the ROI. After selection of the ROI, the sliding mirror is pulled out of the quadrocular adapter and light travels straight toward the spectrograph and the detector camera (Path 2 in Fig. 3.1). Scanning could begin after the sliding mirror is pulled out. The spectrograph is used for the dispersion of light and the detector camera is used to record the spatial-spectral information.

3.3 Operating principle

The operating principle is similar to that as mentioned above in the line-scanning imager in Sec. 2.2.3.1. In addition, this setup includes a video camera.

The integration of a video camera into the pushbroom HSI system makes it a more efficient and flexible imaging scheme. It allows users to view the sample, and from there a ROI is selected from which spectral information is acquired. This ROI can be smaller than the FOV of the video camera. Although the spectral information can first be acquired from the entire FOV, and only those from the ROI can be extracted from the datacube later. However by doing so, the system has taken many more scans from outside the ROI. This increases both the data acquisition time and the data file size to be handled.

Fig. 3.2: Photograph and detailed schematic diagram of pushbroom HSI system.

The addition of a video camera to the setup requires position calibration between the two cameras, as they have different views of the sample. The video camera looks at a rectangular area of the sample, while the detector camera has a line of view (LOV) across the sample. The length of the detector camera's LOV is also longer than the width of the video camera's FOV. The actual views of the detector and video cameras can be seen in Fig. 3.2, where the light rays in green show the scene viewed by the video camera when the sliding mirror is pushed in, while the light rays in red show the view viewed by the detector camera when the sliding mirror is pulled out. The components of the spectrograph in Fig. 3.2 do not represent the components in the actual spectrograph.

3.4 Calibrations of pushbroom HSI system

The calibration can be divided into three main parts (FOV, spectral, and position).

3.4.1 FOV calibration

Cal_{FOV} (mm) refers to the length of the FOV of the video camera in the vertical direction.

At the minimum and maximum zooms (adjusted using the fine focus adapter), Cal_{FOV} was

measured to be 5.17 mm and 4.32 mm, respectively. This was done by first placing a sample onto the stage. The stage was displaced by a distance to move the sample's reference point from the top to the bottom of the FOV of the video camera. This stage displacement was Cal_{FOV} . The results presented in the following sections of this chapter are all at maximum zoom where Cal_{FOV} was measured to be 4.32 mm.

3.4.2 Spectral calibration

The spectrum from each sample point along the detector camera's LOV is dispersed by the spectrograph. Each spectrum spreads along the y-axis of the detector camera. This calibration assigns each row of the sensor array of the detector camera (DC_Y) of to a specific wavelength band. Calibration was carried out by imaging a flat sample illuminated by 12 calibration wavelengths (WL_{Cal}) (470 nm and 500 nm - 1000 nm with 50-nm incremental steps) from a tunable laser source (SuperK Select 4xVIS/IR, SuperK Select-/nIR1, SuperK Extreme EXR-15, NKT Photonics). As each wavelength band from the source has a certain bandwidth, the DC_Y with the highest intensity for each calibration wavelength was recorded. Fig. 3.3 shows the calibration using a 700 nm WL_{Cal} resulting in a DC_Y of 484.

Fig. 3.3: Image from detector camera during spectral calibration of 700 nm.

A second-order polynomial model was used to relate each DC_Y to its calibration wavelength and is shown in Eq. (3.1), where a , b , and c are constants. Subsequently, a second-order polynomial regression model was used to determine the values of a , b , and c , which were found to be $a = 7.34536 \text{ E-5 nm}$, $b = 0.725977 \text{ nm}$, and $c = 331.871 \text{ nm}$. With these constants, each DC_Y was assigned a wavelength.

$$WL_{Cal} = a \cdot DC_Y^2 + b \cdot DC_Y + c. \quad (3.1)$$

3.4.3 Position calibration

As both the cameras in the pushbroom HSI system have different views of the sample (Sec. 3.3), a two-step position calibration is carried out so that a relationship between the different views of the sample by the two cameras can be drawn.

3.4.3.1 Cal_L and Cal_R

This calibration was done as the width across the sample viewed by the video camera was shorter than the detector camera. Cal_L and Cal_R refer to the columns of the sensor array of the detector camera (DC_X) corresponding to the extreme left and right views of the video camera, respectively (Fig. 3.4). The sample used was a United States Air Force (USAF) chart, placed such that the left edge of a dark square was along the extreme left view of the video camera. By looking at the detector camera image, the position of the dark square is easily identified. The DC_X which corresponded to the left side of the dark portion was Cal_L . This process is shown in Fig. 3.5. Cal_L was found to be 224, which means that the left most view of the video camera was imaged onto the 224th column of the sensor array of the detector camera. Cal_R was obtained using similar procedure and was found to be 777.

Fig. 3.4: Definition of Cal_L and Cal_R.

Fig. 3.5: Cal_L calibration.

3.4.3.2 Cal_{LOV}

This calibration was done to determine the row of the sensor array of the video camera (VC_Y) which shared the same view as the LOV of the detector camera (Fig. 3.6). Cal_{LOV} was found by first looking at the detector camera view and then slowly changing the sample's position until a change on the detector camera view was observed. This happened when the sample enters the LOV of the detector camera. Cal_{LOV} was found to be 542. The

detector camera had an LOV across the sample which corresponded to the 542th row from the top of the sensor array of the video camera (Fig. 3.7).

Fig. 3.6: Definition of Cal_{LOV} .

Fig. 3.7: Cal_{LOV} calibration.

3.5 User-defined parameters

These parameters give the user flexibility in using the system so that it can be faster and give only the required data for later analysis.

3.5.1 Region of interest

The user-selectable ROI determines the sample region within the video camera's FOV from which the data are collected and stored. Selection is done by simply dragging a rectangular area across the FOV. The ROI is described by four parameters; "Top, Bottom, Left, and Right," as shown in Fig. 3.8. "Top and Bottom" refer to the VC_Y which correspond to the top and bottom of the ROI, respectively. "Left and Right" refer to the columns of the sensor array of the video camera (VC_X) which correspond to the extreme left and right views of the ROI, respectively. A shorter ROI (vertical direction) can result in fewer scans, thus reducing data acquisition time and data size. A narrower ROI (horizontal direction) will not reduce the data acquisition time but will reduce the data size.

Fig. 3.8: Definition of "top, bottom, left and right."

3.5.2 Spectral range

Both the detector camera and spectrograph have the same spectral range of 400 nm - 1000 nm. Therefore the maximum spectral range of the integrated system is also the same. The user selected spectral range is defined using WL_{Min} (nm) and WL_{Max} (nm), which depends on the illumination source and the spectral range of interest. Spectral information beyond this range will not be recorded. A smaller spectral range results in a smaller data size but will not affect the acquisition time.

3.5.3 Stage step size

The pushbroom HS imager scans the ROI from top to bottom sequentially. The distance that the y-axis stage moves in each step between subsequent scans is defined by “Step.” For example, when Step is set to 5, the y-axis stage will move by a distance imaged by five rows of the sensor array of the video camera. A bigger Step results in a shorter acquisition time and can give a poorer spatial resolution along the y-axis. Thus, Step has to be adjusted to give a good balance between data acquisition time and spatial resolution along the y-axis.

3.5.4 Settings of detector camera

The exposure time and electron-multiplying (EM) gain of the detector camera can be adjusted depending on the illumination condition. A high EM gain is used in low-intensity illumination conditions to increase the sensitivity of the detector camera. However, when the EM gain is set at a value which is too high, it can lead to pixel saturation of the sensor of the detector camera. Both the EM gain and exposure time have to be optimised to reduce exposure time while still getting high quality images from the detector camera. This will minimise the overall data acquisition time.

3.6 Return values and vectors

The steps and procedures mentioned in Sec. 3.4 and Sec. 3.5 are used to produce four return values and two vectors. They are used to control the detector camera and y-axis stage to collect data according to the user-defined parameters.

3.6.1 X_{Min} and X_{Max}

X_{Min} and X_{Max} refer to the DC_X which correspond to the Left and Right of the ROI, respectively. Each scan records data from the detector camera between X_{Min} and X_{Max} only. VC_X and DC_X are akin to different scales while referring to the same object (Fig. 3.9).

Linear interpolation is used to determine the values of X_{Min} and X_{Max} using Eq. (3.2) and Eq. (3.3), respectively. The Cal_L and Cal_R mentioned in Sec. 3.4.3.1 are used here. The detector camera does not recognise X_{Max} . It requires the starting column index X_{Min} and the length of column X_{Length} , which is calculated using Eq. (3.4).

Fig. 3.9: Definition of X_{Min} and X_{Max} .

$$X_{Min} = rd \left[\frac{Left-1}{1600-1} \cdot (Cal_R - Cal_L) + Cal_L \right], \quad (3.2)$$

$$X_{Max} = rd \left[\frac{Right-1}{1600-1} \cdot (Cal_R - Cal_L) + Cal_L \right], \quad (3.3)$$

$$X_{Length} = X_{Max} - X_{Min} + 1, \quad (3.4)$$

where rd means round off to nearest integer.

3.6.2 WL vector

WL is a vector which assigns a wavelength to each DC_Y . WL is calculated using Eq. (3.5). The constants a , b , and c obtained in Sec. 3.4.2 for spectral calibration are used here.

$$WL = a \cdot DC_Y^2 + b \cdot DC_Y + c. \quad (3.5)$$

3.6.3 Y_{Min} and Y_{Max}

Y_{Min} and Y_{Max} refer to the DC_Y which correspond to the WL_{Min} and WL_{Max} of the selected spectral range, respectively. In each scan, only data between rows Y_{Min} and Y_{Max} of the sensor array of the detector camera are recorded. The constants a , b , and c from the spectral calibration in Sec. 3.4.2 are used here. Y_{Min} and Y_{Max} are determined using the real

solution of a quadratic equation. Equation (3.6) is formed by rearranging Eq. (3.1) for WL_{Min} . The real solution to Eq. (3.6) is used to calculate Y_{Min} using Eq. (3.7). Similarly, Y_{Max} is calculated using Eq. (3.8). The detector camera does not recognise Y_{Max} . It requires the starting row index Y_{Min} and the length of row Y_{Length} , which is calculated using Eq. (3.9).

$$a \cdot DC_Y^2 + b \cdot DC_Y + (c - WL_{Min}) = 0, \quad (3.6)$$

$$Y_{Min} = rd \left[\frac{-b + \sqrt{b^2 - 4a(c - WL_{Min})}}{2a} \right], \quad (3.7)$$

$$Y_{Max} = rd \left[\frac{-b + \sqrt{b^2 - 4a(c - WL_{Max})}}{2a} \right], \quad (3.8)$$

$$Y_{Length} = Y_{Max} - Y_{Min} + 1. \quad (3.9)$$

The spectrograph has the same spectral range as the detector camera, which is 400 nm - 1000 nm. Thus the maximum spectral range of this system is also 400 nm - 1000 nm. The maximum Y_{Length} is calculated to be 756. This means that the pushbroom HSI system detects 756 wavelength bands within a spectral range of 400 nm - 1000 nm. Using the chosen definition of spectral imaging from Fresse *et al.* [48], this system is classified as a HS imager. The average spectral gap between adjacent bands is about 0.795 nm.

3.6.4 Stage position vector

This vector represents the positions of the y-axis stage that it needs to be during scanning so that only the ROI is scanned from its top to bottom at a stage step specified by the user. The vector is calculated from the home position of the y-axis stage. Cal_{FOV} from Sec. 3.4.1 and Cal_{LOV} from Sec. 3.4.3.2 are needed.

The relationship between the count and displacement of the y-axis stage (CD) was determined to be about 116508.4 counts/mm using the specifications of the y-axis stage in Eq. (3.10).

$$CD = \frac{\text{Gear ratio}}{\text{Thread pitch}} \cdot \text{Sensor resolution} = \frac{28.44444 \text{ rev}}{0.5 \text{ mm}} \cdot 2048 \frac{\text{counts}}{\text{rev}} \approx 116508.4 \text{ counts/mm.} \quad (3.10)$$

Prior to the first scan, the y-axis stage shifts the sample until the top of the ROI is in line with the detector camera's LOV. This displacement in millimeters is calculated using top , Cal_{LOV} , and Cal_{FOV} . This is later converted to displacement in counts of the y-axis stage using CD . By adding this to the current y-axis stage position in counts (Y_{Pos}), the position of the y-axis stage in counts for the first scan ($\text{Pos}_{\text{Start}}$) can be calculated using Eq. (3.11). Similarly, the position of the final scan (Pos_{End}) can be calculated from Eq. (3.12). The y-axis stage is closer to its home position during the first scan compared to the last scan (Fig. 3.10). Thus, $\text{Pos}_{\text{Start}}$ is smaller than Pos_{End} . The step in counts of the y-axis stage (Step_{Cts}) is calculated based on the user-defined Step , Cal_{FOV} , and CD using Eq. (3.13).

$$\text{Pos}_{\text{Start}} = \frac{\text{Top} - \text{Cal}_{\text{LOV}}}{1200} \cdot \text{Cal}_{\text{FOV}} \cdot CD + Y_{\text{Pos}}, \quad (3.11)$$

$$\text{Pos}_{\text{End}} = \frac{\text{Bottom} - \text{Cal}_{\text{LOV}}}{1200} \cdot \text{Cal}_{\text{FOV}} \cdot CD + Y_{\text{Pos}}, \quad (3.12)$$

$$\text{Step}_{\text{Cts}} = \frac{\text{Step}}{1200} \cdot \text{Cal}_{\text{FOV}} \cdot CD. \quad (3.13)$$

Fig. 3.10: Positions of y-axis stage and ROI as scanning progresses.

A vector representing the y-axis stage position in counts for each scan is tabulated from $\text{Pos}_{\text{Start}}$ to Pos_{End} , with increment of Step_{Cts} . It is then rounded off to the nearest integer, which will be used to control the y-axis stage. The vector length is also the number of scans needed for the chosen ROI and Step .

3.6.5 Significance of return values and vectors

X_{Min} and X_{Max} are related to the location of the ROI in the x -direction. Y_{Min} and Y_{Max} refer to the user-defined spectral range. These four values together form a corresponding region on the detector camera sensor array from which data are recorded in each scan. Each scan produces a two-dimensional data in the spatial-spectral domain, before the stage moves on to the next position. This process is repeated until scanning takes place at all the positions indicated by the stage position vector.

3.7 HyperSpec

A software based on LabVIEW® called HyperSpec was developed in-house. The control panel is shown in Fig. 3.11. It is used for the software interfacing of the three-axis stage and detector and video cameras, and incorporates all the points discussed in Sec. 3.4-Sec. 3.6. After calibration and entering the user-defined parameters, the scanning can begin. The return values are determined automatically, and the repeating process of stage movement and then detector camera data recording will also run on its own. After all the scanning has been completed, the stage places the sample back to the same position just before scanning started. User will then decide whether and where to save these files. The software protocol of HyperSpec is shown in Fig. 3.12.

Fig. 3.11: HyperSpec control panel.

Fig. 3.12: HyperSpec software protocol.

3.8 Data processing and visualization

The saved files are imported and processed by an in-house written script in MATLAB®. The script arranges the two-dimensional data to a single three-dimensional datacube (Appendix A). As data representation is more flexible and can vary depending on the needs, more parameters can be altered and customised. Many types of plots can be made available, such as spectrum plot, images at different wavelength bands and datacube.

3.9 Results and discussion

The measurements in this section were taken at maximum zoom where the full FOV of the video camera was about $4.32 \times 5.76 \text{ mm}^2$ with working distance of about 21.5 cm.

3.9.1 Video camera for selectable ROI

A USAF resolution chart was used for this section. A fiber-optic pigtailed source (MI-150, Edmund Optics) was used for illumination. The full FOV of the video camera before measurement and the selected ROI of the Group 3 of the USAF chart as indicated by a black rectangle can be seen in Fig. 3.11. This section first shows the different plots that can be acquired from each set of data (Fig. 3.13-Fig. 3.15). The MATLAB® script to plot a cut-datacube as in Fig. 3.14(a) is shown in Appendix B.

By comparing the selected ROI to the intensity mapping captured by the system at a particular wavelength, it can be used to validate whether the system is working well and capturing data only from the ROI. Fig. 3.16 is made up of an image of the ROI, with two intensity mappings at 650 nm placed beside and below the ROI. The four dashed lines in this figure match features in the ROI to the same features in the intensity mappings. It is observed that the system scanned only across the selected ROI, and only data in the ROI were saved. This validates the steps and formulas mentioned in Sec. 3.4-Sec. 3.8. The longer vertical dotted line also shows that the ROI and data have the same orientation. Therefore, the y-axis stage and detector and video cameras are all aligned with respect to each other. The video camera is successfully integrated in the pushbroom HS imager for a user-selectable ROI to minimise data acquisition time and data size.

Fig. 3.13: (a) Sequence of data acquisition and (b) datacube.

Fig. 3.14: (a) Cut-datacube and (b) wavelength stack of bands 550:25:750 nm.

Fig. 3.15: Intensity mappings of nine selected spectral bands.

Fig. 3.16: Comparison of ROI and intensity mappings.

3.9.2 Lateral resolution

This section uses the same set of data as in Sec. 3.9.1. From Fig. 3.17, the horizontal and vertical lines of Group 3 Element 5 (G3E5) can still be distinguished. Thus, the lateral resolutions of the system along the horizontal and vertical directions at 650 nm are determined using G3E5 of the USAF chart. The lateral resolutions in the basic configuration without any image enhancement are about 40 μm .

Fig. 3.17: (a) ROI and (b) intensity mapping of 650 nm.

3.9.3 Spectral resolution

Fig. 3.18: Spectra of 633-nm and 785-nm wavelength sources.

HS measurements were conducted on a 99% reflectance standard (SRS-99-010, Labsphere) illuminated separately by 633-nm and 785-nm single wavelength sources (1146P, JDS Uniphase and LBX-785-130-CIR-PP, Oxixus) to investigate the spectral resolution of the system. The results are shown in Fig. 3.18 and the full widths at half

maximum of the acquired spectra of 633-nm and 785-nm single wavelength sources are about 3.5 nm and 4.7 nm, respectively.

3.9.4 Reflection imaging of bio-sample

The bio-sample was a chicken breast tissue devoid of fat and skin, with a visible blood clot on the surface. This part of the chicken breast was chosen so that the blood clot could provide a contrast in the image. The sample on the glass slide and the ROI are shown in Fig. 3.19. A white light illumination source (MI-150, Edmund Optics) was used. Fig. 3.20 shows the intensity mappings of four different wavelengths. The regions where 400 spectra were extracted and processed to represent the spectra of the blood clot and the chicken breast tissue are marked by the small white and black rectangles respectively, in Fig. 3.19(b) and Fig. 3.20. Fig. 3.21 shows the processed spectra of the chicken breast tissue and the blood clot which are found to be easily distinguishable from each other. These results indicate that such spectral data can be used as a data library to compare and identify unknown samples in the future.

Fig. 3.19: (a) Chicken breast tissue on glass slide and (b) ROI.

Fig. 3.20: Intensity mappings at (a) 550 nm, (b) 630 nm, (c) 670 nm, and (d) 850 nm.

Fig. 3.21: Spectra of blood clot and chicken breast tissue.

3.9.5 Fluorescence imaging of phantom tissue sample

A Rhodamine 6G fluorescent film which was placed on a phantom tissue sample (Simulab Corporation) and the ROI are shown in Fig. 3.22. An excitation wavelength of 500 nm (SuperK Select 4xVIS/IR, SuperK Extreme EXR-15, NKT Photonics) was used with a beam expander unit so that the expanded beam covered the entire FOV of the video camera. The measurement was taken with an exposure time of 150 ms and an EM gain of 10. The entire spectral range from 400 nm - 1000 nm was recorded.

The intensity mappings of 535 nm, 563 nm, and 585 nm are shown in Fig. 3.23 to illustrate the differences in fluorescence intensity at varying wavelengths. Fig. 3.24 shows the processed excitation and fluorescence spectra, each normalised with respect to itself. A shorter spectral range (400 nm - 800 nm) is shown for a better representation. The orange solid line shows the fluorescence spectrum calculated by averaging the 400 spectra within the region indicated by the black rectangle in Fig. 3.22(b) and Fig. 3.23. The green dotted line shows the excitation spectrum measured separately from a piece of white paper.

Fig. 3.22: (a) Rhodamine 6G fluorescent film on tissue phantom and (b) ROI.

Fig. 3.23: Intensity mappings of (a) 535 nm, (b) 563 nm (peak emission), and (c) 585 nm.

Fig. 3.24: Normalised excitation and fluorescence spectra.

The HSI of fluorescing samples is able to capture multiple fluorescent images at different wavelength bands. In this study, about 250 fluorescent images were captured between 500 nm - 700 nm (three shown in Fig. 3.23). Compared to the use of conventional imaging setup which uses a fluorescence filter to capture all the emission wavelengths in a single image, HSI provides much more information that can be used for a more accurate disease diagnosis. This can prove useful in disease diagnosis of the colon where the intensity and distribution of endogenous fluorophores are indicators of disease progression [20].

3.10 Summary

A pushbroom HS imager which incorporates a video camera not only for direct video imaging (benefits mentioned in Sec. 3.1) but also for a user-selectable ROI within the full imaging FOV of the video camera is proposed and demonstrated in this chapter. These concepts bring several benefits especially to a pushbroom HS imager. After selecting the ROI, scanning takes place only within the ROI. There is no unwanted scanning, thus minimizing the data acquisition time and data size. A smaller data size in turn translates to a shorter computational time in data processing and analysis. Similar applications can also be applied to spectral-scanning and snapshot imagers. However, it will not result in a shorter data acquisition time in spectral-scanning (number of scans depends on number of spectral band, not size of ROI) or a snapshot imager (only one scan required). The use of a video camera for a user-selectable ROI presented in this chapter tries to negate the pushbroom HS imager as being a relatively slower HS imager.

In the current configuration, the video camera has an adjustable full imaging FOV using the fine focus adapter. The minimum and maximum FOV of the video camera are about $4.32 \times 5.76 \text{ mm}^2$ (working distance of about 21.5 cm) and $5.17 \times 6.89 \text{ mm}^2$ (working distance

of about 23.8 cm), respectively. The full FOV is also the maximum size of the ROI that can be selected by the user. The system has a maximum spectral range covering the visible to near-infrared wavelength band from 400 nm - 1000 nm. By using a detector camera and spectrograph suitable for imaging wavelengths more than 1000 nm, it is possible to extend the spectral range further into the infrared wavelengths. The lateral resolution of this system at maximum zoom without using any image enhancement is about 40 μm . Such a lateral resolution makes the system suitable for use in biomedical imaging on tissue. A total of 756 spectral bands can be acquired, which is more than the required when compared to the system's spectral resolution. However, it is still desired to gather spectral information from spectral bands with spectral gap smaller than the resolution. This allows a more detailed collection of spectral information from samples with more detailed spectral signatures to build a spectral library, which can be very useful for diagnostic applications. In cases where the sample's spectral signatures are not as detailed, data binning in the spectral direction can be applied so as to reduce the number of spectral bands without losing spectral details.

In reflection mode imaging, a common quartz halogen white light source (MI-150, Edmund Optics) was used. With respect to the maximum spectral range of interest (400 nm - 1000 nm), the bulb used in this light source had a poor transmittance from 400 nm - 500 nm and 800 nm - 1000 nm. Also within the same spectral region, the detector camera has lower quantum efficiency. This can be seen in the spectral plot from the reflection mode (Fig. 3.21) where intensity counts below 450 nm and above 900 nm are always much lower compared to the central wavelengths. Without changing the detector camera, this issue can be resolved using a light source with a higher intensity in the extreme ends of the full spectral range of interest.

The experiments with the bio- and fluorescent phantom samples shown in this chapter also demonstrate that the developed pushbroom HS imager can be used for both reflection and fluorescence imaging modalities. The lateral resolution can be varied and improved by using additional optical elements and digital schemes. The scope of this system can also be extended to other applications such as cellular-scale biomedical imaging. The HS imager serves as a main platform for probe-based imaging in biological intra-cavities such as colon to detect cancerous tissues by integrating it with a flexible probe scheme.

The following chapter will be elaborating on a first-of-its-kind spatial-scanning HSI probe. This is realised by integrating a flexible imaging probe with the table-top pushbroom HS imager mentioned in this chapter. This extends the scope of application of the developed pushbroom HS imager by enabling it to perform endoscopic imaging.

Chapter 4: Pushbroom hyperspectral imaging probe for bio-imaging applications

The three common methods to perform hyperspectral imaging are the spatial-scanning, spectral-scanning and snapshot methods. However, only the spectral-scanning and snapshot methods have been configured to a hyperspectral imaging probe. This chapter presents a spatial-scanning pushbroom hyperspectral imaging probe, which is realised by integrating a pushbroom hyperspectral imager with an imaging probe. The proposed hyperspectral imaging probe can also function as an endoscopic probe by integrating a custom-fabricated image fiber bundle unit. The imaging probe is configured by incorporating a gradient index lens at the end-face of an image fiber bundle that consists of about 50 000 individual fiberlets. The necessary simulations, methodology and detailed instrumentation aspects are discussed, followed by the assessment of the developed probe's performance. Resolution test target using United States Air Force chart as well as bio-samples using chicken breast tissue with blood clot are used as test samples for resolution analysis and for performance validation. This system is built on a pushbroom hyperspectral imaging system with a video camera (Chapter 3) and has the advantage of acquiring information from a large number of spectral bands with selectable region of interest. The advantages of this spatial-scanning hyperspectral imaging probe can be extended to test samples or tissues residing in regions that are difficult to access with potential diagnostic bio-imaging applications.

4.1 Introduction

Hyperspectral imaging (HSI) has been configured to be used as an optical probe and in some cases as an endoscope to image the body cavity. They have been used to image the vasculature of the lower lip for oxy-hemoglobin studies [21], as well as the skin [65,78] and in otolaryngoscopic investigations [67] for cancer studies. Although there are three commonly used methods to perform HSI, only the spectral-scanning and snapshot methods have been demonstrated in a HSI probe. Both the liquid crystal [65] and acousto-optical tunable filters [78] have been used in spectral-scanning HSI probe, and image mapping spectroscopy has been used in snapshot HSI probe [21]. These HSI probes usually use a

fiber bundle to deliver light from the sample (distal end) to the proximal end where hyperspectral (HS) measurement is performed. One reason why the spatial-scanning method has been left out for such an application is that the mechanical scanning required during HS measurement makes it much slower compared to the other two methods. However, existing HSI probes that use spectral-scanning or snapshot method has so far only provided up to about 50 spectral bands [21,67].

In this context, this chapter details the instrumentation, performance and capability of a pushbroom HSI probe. This fills the gap in translating the spatial-scanning pushbroom HSI system to a pushbroom HSI probe. Also, the advantage of using such a configuration is that information from a large number of spectral bands, of the order of several hundreds, can be acquired. The feasibility and efficiency of the probe system are illustrated by performing HS measurements on a United States Air Force (USAF) resolution chart and chicken breast tissue with blood clot as test samples.

4.2 Instrumentation of pushbroom HSI probe

The pushbroom HSI probe is shown in Fig. 4.1 and is divided into two main parts, the imaging probe and the pushbroom HS imager. The imaging probe is an assembly of an imaging fiber optic bundle and a gradient index (GRIN) lens. The fiber bundle (IGN 11/50, Sumitomo Electric) has an active and an outer diameter of about 1 mm and 1.2 mm, respectively. Attached to the distal end of the fiber bundle is a GRIN lens (GT-IFRL-100-cus-50-NC, Grintech) with a diameter and length of about 1 mm and 4.2 mm, respectively. The GRIN lens was custom-fabricated for $1\times$ magnification with a working distance of 0.3 mm at 532 nm in air at both sides. The fiber bundle and the GRIN lens are attached together using a stainless steel tubing, with an outer diameter and length of 1.6 mm and 25 mm,

respectively. The maximum diameter on the distal end of the imaging probe is only 1.6 mm, due to the stainless steel tubing. This makes the imaging probe suitable to be used as an endoscopic probe for imaging in confined places.

Fig. 4.1: Schematic diagram of pushbroom HSI probe.

The pushbroom HS imager used in this chapter is the same as in Chapter 3. The sample was placed at about 0.3 mm away from the GRIN lens using a 3-axis mechanical stage. A broadband fiber-optic pigtailed source (MI-150, Edmund Optics) was used for sample illumination. The GRIN lens produces the image of the sample on the distal end-face of the fiber bundle. This image is transferred to the proximal end of the fiber bundle and directed towards the doublet forelens (2-50145, Navitar). Before HS scanning, the sliding mirror is initially pushed into the quadrocular adapter and directs light towards the video camera, as shown by Path 1 in Fig. 4.1. The video camera (UI-1550LE-C-HQ, iDS) allows direct video imaging of the proximal end-face of the fiber bundle where the image of the sample is formed. This allows easy positioning of the proximal end of the fiber bundle by the 3-axis motorised stage (x - and y -axis: M-112.2DG, z -axis: M-110.1DG, Physik Instrumente). When the sliding mirror is pulled out of the quadrocular adapter, the image travels towards the spectrograph (V10E, Specim ImSpector) and the detector camera (LucaEM DL-604M-

OEM, Andor) (Path 2 in Fig. 4.1). The spectrograph has a slit allowing only one line of the image to reach the other optical elements in the spectrograph for spectral dispersion. The detector camera records the resulting two-dimensional spatial-spectral information with an integration time of 0.1 s. HS scanning is done by the y-axis motorised stage to carry out sequential line-scanning of the proximal end-face of the fiber bundle with a step size of 3.6 μm . The HS imager detects 756 spectral bands within a spectral range of 400 nm - 1000 nm.

4.3 HyperSpec

HyperSpec is a custom-developed software using LabVIEW® where user can choose a particular region within the field of view (FOV) of the video camera as the region of interest (ROI). This is the same software mentioned in Sec. 3.7. The position of the proximal end-face of the fiber bundle may vary, but as long as it is within the FOV of the video camera, the proximal fiber end-face can be selected as the ROI. HyperSpec controls the y-axis stage to perform sequential HS measurement. Such flexibility allows easy positioning of the proximal fiber end-face of the fiber bundle and makes the system alignment convenient. HyperSpec takes into account the calibrations of the system, user-defined parameters, and the subsequent hardware synchronization during HS line-scanning measurement.

4.4 GRIN lens

A GRIN lens was used as a miniature objective lens so that the image of the sample falls onto the distal end-face of the image fiber. The GRIN lens was initially designed for use with 532 nm light source with a working distance of 0.3 mm, considering only the on-axis optical performance. Using Zemax simulation, the length of the GRIN lens was determined to be about 4.2 mm. The GRIN lens was attached to the end-face of the fiber bundle by Grintech to form the imaging probe.

In order to use this fiber probe for HSI, more factors are considered. The most important factor includes the spectral range of interest from 400 nm - 1000 nm and the on- and off-axis optical performances. Five equally weighted wavelengths (400 nm, 550 nm, 700 nm, 850 nm and 1000 nm) were used in the simulation to represent the spectral range of interest from 400 nm - 1000 nm. Three field positions were considered, on-axis, 0.32 mm (0.707 full-field) and 0.45 mm (full-field). Using Zemax's default merit function (type: RMS, criteria: wavefront, reference: centroid, pupil integration: Gaussian quadrature with three rings and six arms), the optimised object-lens distance was found to be about 0.316 mm.

At the optimised object-lens distance of about 0.316 mm, it is observed from Fig. 4.2 that each wavelength behaves differently as it moves through the GRIN lens. This causes wavelength-dependent optical characteristics on the distal end-face of the fiber bundle. It is evident from Fig. 4.2 that the focus positions shift away from the object as wavelength increases, causing the images formed at the distal end-face of the fiber bundle to be wavelength-dependent. The on-axis root-mean-square radii of the spot diagrams with centroid references for 400 nm, 550 nm, 700 nm, 850 nm and 1000 nm are 41.440 μm , 2.511 μm , 14.872 μm , 24.018 μm and 29.415 μm , respectively. The spot diagrams of the three field positions of 550 nm and 1000 nm at the optimised object-lens distance are shown in Fig. 4.3 and Fig. 4.4, respectively. The spot diagrams of 400 nm, 700 nm and 850 nm are in Appendix C. It can also be seen that the amount of light collected by the GRIN lens decreases as the object is positioned further away from the centre of the lens. This may cause the features collected from the peripheral of the fiber bundle to be suppressed by the high intensity of light collected from the centre.

Fig. 4.2: Optimised layout of GRIN lens at five representative wavelengths.

Fig. 4.3: Zemax spot diagram of 550 nm on distal end-face of fiber bundle.

Fig. 4.4: Zemax spot diagram of 1000 nm on distal end-face of fiber bundle.

Although the GRIN lens is useful as a miniature objective lens, a detailed study on the use of such a GRIN lens in a HSI probe is important as this can affect the spatial resolutions of the spectral images captured by the system. At the optimised object-lens distance of about 0.316 mm, the Zemax results show that the on-axis root-mean-square radii with centroid references of the spot diagrams for the different wavelengths have very large variations from 2.511 μm - 41.440 μm , due to chromatic aberration in the GRIN lens. A good GRIN lens to be used for HSI with a wide spectral range should be able to minimise chromatic aberration so that the variations in the spatial resolutions at different wavelengths are reduced. In conventional imaging setups using lenses, a doublet can be used to correct for chromatic aberrations and similar strategy may be applied in GRIN lens. Other methods that can be explored to correct for chromatic aberrations in the distal end of the HSI probe include using miniaturised doublets or lens systems.

4.5 Data processing

The data were processed off-line using MATLAB®. The results presented in this chapter are not in terms of intensity but were referenced to take into account the non-uniform collection of light by the GRIN lens and any uneven illumination of the sample.

HS measurement of the USAF chart shows the system's performance and this was done in transmission mode. In order to get the transmittance data, the sample data were corrected by dark reference (Dark) and white reference (White) using Eq. (4.1). Sample data were acquired when the bars on the USAF resolution chart were imaged. Dark data were acquired when the light source was turned off and the forelens covered. It represents the image with dark current noise where the transmittance was 0%. White data were acquired by imaging a clear region of the USAF resolution chart where the transmittance was taken to be 100%. x and y refer to the orthogonal spatial dimensions and λ refers to the spectral band. Smooth is the 11-point moving average in the spectral direction for spectrum smoothing.

$$\text{Transmittance}(x, y, \lambda) = \text{Smooth} \left[\frac{\text{Sample}(x, y, \lambda) - \text{Dark}(x, y, \lambda)}{\text{White}(x, y, \lambda) - \text{Dark}(x, y, \lambda)} \right]. \quad (4.1)$$

HS measurement of the chicken breast tissue with blood clot shows the system's capability to acquire biological images in reflection mode, when there is access to only one side of the sample. In order to get the reflectance data, the sample data were corrected by dark reference (Dark) and white reference (White) using Eq. (4.2). Sample data were acquired by imaging the chicken breast tissue. Dark data were acquired when the light source was turned off and the forelens covered. It represents the image with dark current noise where the reflectance was 0%. White data were acquired by imaging a 99% reflectance standard (SRS-99-010, Labsphere) where the reflectance was taken to be 99%.

$$\text{Reflectance}(x, y, \lambda) = \text{Smooth} \left[\frac{\text{Sample}(x, y, \lambda) - \text{Dark}(x, y, \lambda)}{\text{White}(x, y, \lambda) - \text{Dark}(x, y, \lambda)} \right] \times 0.99. \quad (4.2)$$

4.6 Results and discussion

The results of the HS measurements using the USAF resolution chart to determine the imaging characteristics of the system are shown in this section. The HS results of the reflectance imaging of the bio-sample are also included.

4.6.1 Scale and orientation

Fig. 4.5: Comparison of ROI and intensity mappings of USAF chart G2E4.

Fig. 4.5 shows the image of the selected ROI, which is the vertical bars of Group 2 Element 4 (G2E4) of the USAF chart, and two intensity mappings of 660 nm from the datacube, placed beside and below the ROI. The circular dashed lines indicate the position of the imaged proximal end-face of the fiber bundle within the ROI. Data outside the circular dashed lines were the background and thus ignored. The four straight dashed lines match features in the ROI to the same features in both the intensity mappings. The longer

horizontal dashed line also shows that the ROI and the HS data have the same orientation. These show that both the horizontal and vertical scales between the ROI and HS were the same and that the system was properly aligned and calibrated.

4.6.2 Effective FOV

The horizontal bars of G1E6 of the USAF chart were imaged, as shown in Fig. 4.6. They were specifically chosen as they fit nicely within the image of the fiber bundle as captured by the video camera. The results in Fig. 4.6(b) show that the entire end-face of the fiber bundle can be used to capture HS data, utilising the maximum FOV achievable by the distal end of the probe.

Fig. 4.6: (a) ROI and (b) intensity mapping of horizontal bars of USAF chart G1E6.

4.6.3 Lateral resolution

Group 3 of the USAF chart was imaged in three separate measurements, as the whole of Group 3 could not be imaged in one measurement. The results are shown in Fig. 4.7. Using the 505-nm intensity mapping, the vertical and horizontal bars of G3E5 [Fig. 4.7(f)] can still be distinguished. Therefore the vertical and horizontal lateral resolution of this system at 505 nm is evaluated to be about 40 μm .

Fig. 4.7: Images of USAF chart Group 3. ROIs of (a) G3E1 and G3E2, (b) G3E3 and G3E4, (c) G3E5 and G3E6, 505-nm intensity mappings of (d) G3E1 and G3E2, (e) G3E3 and G3E4, and (f) G3E5 and G3E6.

As a representation, nine out of 756 intensity mappings of G3E5 and G3E6 are selected from the entire spectral range (400 nm - 1000 nm) and shown in Fig. 4.8. It can be seen that some features, more evidently the vertical bars of G3E5, do not appear to be the same in all spectral bands. They appear to be sharper at 505 nm and 570 nm, but became worse as wavelength increases. The HS data in Fig. 4.8 show that the system's lateral resolution is wavelength-dependent. This result coincides with the Zemax simulation results of the GRIN lens where the path of each wavelength varies within the GRIN lens, resulting in different optical performance of the wavelengths on the distal end-face of the fiber bundle. Also, at an object-lens distant of about 0.3 mm, intensity mappings from around 505 nm will have better spatial resolutions compared to other spectral bands.

Fig. 4.8: Nine selected intensity mappings of USAF chart G3E5 and G3E6.

4.6.4 Reflectance imaging of bio-sample

A chicken breast tissue with blood clot was used as the bio-sample to demonstrate the imaging capability of the integrated HSI probe. The sample and the ROI of the measurement are shown in Fig. 4.9.

Fig. 4.9: (a) Sample of chicken breast tissue with blood clot and (b) ROI.

The datacube of this measurement has a size of $100 \times 293 \times 756$ (x , y , λ), and a cut-datacube is shown in Fig. 4.10 to reveal the internal features of the datacube. The intensity mappings of four selected spectral bands are shown in Fig. 4.11. The spectra of the blood clot and the chicken breast tissue were acquired from three regions each, as indicated by the red and black boxes respectively, in Fig. 4.9(b) and Fig. 4.11. Each region is about $0.1 \times 0.1 \text{ mm}^2$ and corresponds to $30 \text{ y-pixel} \times 10 \text{ x-pixel}$ in the datacube. The 900 spectra in the three regions of the blood clot and chicken breast tissue were averaged to give the representative spectra. The centre white lines in the plots shown in Fig. 4.12 are the representative spectra, while the black areas surrounding the white lines represent the standard deviations. The average standard deviations of the reflectance spectra of the chicken breast tissue and blood clot are about $\pm 1.5\%$ and $\pm 1.9\%$, respectively. The spectral results show that the integrated HSI probe is able to acquire the distinct spectra from different parts of the sample reliably. Such spectral data can be stored in a data library and used for the classification and quantification of other similar samples.

Fig. 4.10: Cut-datacube of chicken breast tissue with blood clot.

Fig. 4.11: Four selected intensity mappings of chicken breast tissue with blood clot.

Fig. 4.12: Mean reflectance spectra (white lines) and standard deviation (black areas) of chicken breast tissue and blood clot.

The proposed and illustrated HSI probe has given a new dimension to table-top spatial-scanning HS imagers to perform endoscopic imaging with the addition of an imaging probe. The proposed concept of the spatial-scanning pushbroom HSI probe offered many advantages compared to other existing pushbroom HSI systems which can only perform table-top imaging [54,58,127,128]. Using a video camera in this proposed system offers

flexibility in positioning the proximal end-face of the fiber bundle and allows convenient system alignment. It also gives a colour image of the ROI so that a visual comparison can be made between the colour image and the HS data. The system detects 756 spectral bands within the spectral range from 400 nm - 1000 nm. With this arrangement, existing table-top pushbroom HS imager can be made to perform both table-top and endoscopic imaging, making it suitable for more diagnostic bio-imaging applications such as endoscopy in the gastrointestinal tract and pharynx.

4.7 Summary

A pushbroom HSI probe based on spatial-scanning method have been proposed, optically configured and demonstrated for the first time. The imaging probe is an assembly of a GRIN lens and an imaging fiber optic bundle. The probe delivers the image of the sample to the proximal fiber end-face of the fiber bundle for HS measurement. The system offers 756 spectral bands for detection within the full spectrum range of the system. Lateral resolution of the system is wavelength-dependent and this is in agreement with both the theoretical simulation using Zemax and the follow up experimental validation. The lateral resolutions along the horizontal and vertical directions at 505 nm are about 40 μm . In order to demonstrate the diagnostic bio-imaging capability as a proof of concept, a chicken breast tissue with blood clot was used as test sample. Distinct reflectance spectra of the chicken breast tissue and blood clot were acquired for analysis.

The pushbroom HSI probe can be used on samples that are difficult to reach and close to being stationary. The main advantage is that it can provide hundreds of spectral images. It is envisaged that the hundreds of spectral images that are available for efficient analysis can contribute to potential diagnostic bio-imaging applications in the near future. The scope of

the application of the developed table-top pushbroom HS imager has been extended by enabling it to perform endoscopic imaging using a flexible imaging probe. This configuration can then be used for endoscopic bio-imaging applications, which can be used to image the colon for the detection of cancer.

Existing HSI probes capture images from at most 48 spectral bands within the visible spectrum from about 400 nm - 700 nm [21,52,65,78], while the pushbroom HSI probe presented in this chapter captures images from more wavelengths of 756 spectral bands and a larger spectral range from 400 nm - 1000 nm. With images from more spectral bands, the pushbroom HSI probe can build a more detailed spectral library and the spectral range which also covers the near-infrared enables more information to be acquired. Unlike pushbroom and snapshot HSI probes, spectral-scanning HSI probes have the flexibility to determine the number of spectral bands to capture. In situations where it is required to capture images from lesser number of spectral bands, this flexibility allows spectral-scanning HSI probes to form datacubes at a faster rate.

The subsequent chapter involves a four-dimensional snapshot HS video-endoscope. This is achieved by integrating a flexible two-dimensional to one-dimensional fiber bundle with the table-top pushbroom HS imager mentioned in Chapter 3. The snapshot HS video-endoscope can detect significantly more wavelength bands than existing similar systems.

Chapter 5: A four-dimensional snapshot hyperspectral video-endoscope for bio-imaging applications

Hyperspectral imaging has proven significant in bio-imaging applications and it has the ability to capture up to several hundred images of different wavelengths offering relevant spectral signatures. To use hyperspectral imaging for in vivo monitoring and diagnosis of the body cavities, a snapshot hyperspectral video-endoscope is required. However, such reported systems provide only about 50 wavelengths. A four-dimensional snapshot hyperspectral video-endoscope with a spectral range of 400 nm - 1000 nm has been developed. It can detect 756 wavelengths for imaging, significantly more than such systems. Capturing the three-dimensional datacube sequentially gives the fourth dimension. All these are achieved through a flexible two-dimensional to one-dimensional fiber bundle. The potential of this custom-designed and fabricated compact biomedical probe is demonstrated by imaging bio- and phantom tissue samples in reflectance and fluorescence imaging modalities. It is envisaged that this novel concept and developed probe will contribute significantly towards diagnostic in vivo biomedical imaging in the near future.

5.1 Introduction

Hyperspectral imaging (HSI) was first used in airborne and spaceborne vehicles for the observation of Earth [40]. Its ability to capture data to form a datacube consisting of hundreds of images from contiguous and narrow spectral bands for further analysis has since led to many other applications. These include astronomy [75,129], examination of historical murals [130], quality assessment of food [42], and bio-imaging [131,132]. In biomedical imaging, endoscopes have been developed to image sites within the body that are not easily accessible by conventional table-top setup. HSI has been incorporated into such applications using the spectral-scanning [65,67] and snapshot methods [21]. In real-time endoscopic applications such as *in vivo* disease diagnosis and surgical monitoring, the

snapshot methods are the preferred choice. However, one major drawback of existing snapshot hyperspectral (HS) endoscopes is that only about 50 spectral bands can be acquired [21].

In this context, a snapshot HS probe which can be used for endoscopic bio-imaging applications has been developed. The custom-fabricated probe is flexible along its length and its distal end has a small profile so that it can be inserted into the orifice of the body cavities such as the anus to investigate the gastrointestinal tract. It is a two-dimensional (2-D) array (10×10) of hexagonally-packed optical fiberlets (individual fibers) arranged in rows and columns, which are orderly rearranged to form a one-dimensional (1-D) array (1×100) of fiberlets at the other end. It is to be mentioned that though the use of such 2-D to 1-D fiber bundle has been previously reported, it was only used as an optical element in a field or table-top systems [73,74,76]. These systems using 2-D to 1-D fiber bundles do not have a flexible, long and small probe suitable for endoscopic applications.

In this chapter, the use of a custom-fabricated flexible 2-D to 1-D fiber bundle as a compact four-dimensional (4-D) snapshot HS video-endoscope is illustrated for bio-imaging applications. It forms an image of the sample covering about $1.11 \times 1.32 \text{ mm}^2$ of 100 spatial points at a frame rate of about 6.16 Hz. The spectral range of interest is 400 nm - 1000 nm with 756 spectral bands.

5.2 Instrumentation of HS video-endoscope

The snapshot HS endoscopic probe shown in Fig. 5.1 was developed and installed in-house. This has two main parts, the HS imager and the 2-D to 1-D fiber bundle probe. The HS imager is the major part of a pushbroom HS imager which has been reported in Chapter 3, but without the 3-axis motorised stage. During hardware installation, the video camera

(UI-1550LE-C-HQ, iDS) and the detector camera (LucaEM DL-604M-OEM, Andor) were positioned to produce focused images at the same time. The doublet forelens (2-50145, Navitar) is kept in a fine focus adaptor (2-16265, Navitar). This adaptor is attached to bottom side of the quadrocular adaptor (Y-QT, Nikon), which houses a sliding mirror. The sliding mirror is pushed into the quadrocular adaptor and directs light towards the video camera (Path 1 in Fig. 5.1). The video camera allows direct video imaging and is used to position the 1-D end of the fiber bundle. With the 1-D end of the fiber bundle in place, the sliding mirror is pulled out of the quadrocular adaptor so that light can travel straight towards the spectrograph (ImSpectors V10E, Specim) and the detector camera (Path 2 in Fig. 5.1). The spectrograph disperses the light and the detector camera records the information required to build a datacube with each scan. A broadband light source (MI-150, Edmund Optics) was used for transmittance and reflectance imaging, while a 532-nm diode-pumped solid-state laser are used for fluorescence imaging. The samples are illuminated using two flexible light-guides. During reflectance and fluorescence imaging, the light guides are placed in front of the sample (Fig. 5.1), but they are placed behind the sample during transmittance imaging. During fluorescence imaging, a 550-nm long-pass filter (FEL0550, Thorlabs) is kept before the forelens. Spectral calibration is carried out to find out that the system detects 756 spectral bands within 400 nm - 1000 nm.

The 2-D to 1-D fiber bundle was custom-fabricated by Polymicro TechnologiesTM according to author's specifications (Fig. 5.2). The 100 optical fiberlets in the fiber bundle have core and buffer diameters of 100 μm and 125 μm , respectively (FVP100110125, Molex). They are arranged in a 10 $\times$ 10 hexagonally-packed fashion in the 2-D end and numbered from 1 to 100 across the column towards the right and then down the row [Fig.

5.3(a)]. The vertical and horizontal core-to-core spacings on the 2-D end-face are about 110 μm and 125 μm , respectively. The fiberlets are rearranged by row and column in a 1×100 fashion in the 1-D end and numbered correspondingly from 1 to 100 towards the right [Fig. 5.3(b)]. The core-to-core spacing is about 125 μm . Fiberlet 4 is damaged and found to be inactive as indicated by the dark spot shown in Fig. 5.3, thus it cannot be used for imaging. The flexible bundle has a length of about 1 m with a 3.5 mm diameter polyvinyl chloride jacket. The optical fiberlets in the 1-D end are encased in a stainless steel holder with an end-face area of $5 \times 20 \text{ mm}^2$ and length of 30 mm. While in the 2-D end, they are enclosed in a cylindrical stainless steel holder with a diameter of 5 mm and length of 30 mm.

Fig. 5.1: Instrumentation of snapshot HS video-endoscope.

Fig. 5.2: Photograph of 2-D to 1-D fiber bundle.

Fig. 5.3: Photograph of (a) 2-D and (b) 1-D end-faces showing all fiberlets.

The full line-of-view of the HS imager was measured to have a length of about 12.5 mm. After selecting the optical fiberlet having a buffer diameter of 125 μm , it was estimated that the HS imager would only image about 100 optical fiberlets when placed in a straight line. Hexagonally-packed optical fiberlets result in a bundle with a higher packing ratio compared to one which is packed in a square array. Therefore 100 optical fiberlets were used to make the fiber bundle and arranged in a hexagonally-packed fashion in the 2-D end.

5.3 Operating principle

The 2-D to 1-D fiber bundle allows the 2-D image to be captured by 2-D end of the fiber bundle and be reduced from two spatial dimensions to only one dimension at its 1-D end [133]. The light from the 1-D end of the fiber bundle enters a spectrograph which disperses the light to be detected by the 2-D sensor array. This 2-D to 1-D fiber bundle reduces the three-dimensional (3-D) data (spatial-spatial-spectral) to a 2-D data (spatial-spectral) so that the 2-D sensor array takes only one scan to capture all the information. Capturing the data sequentially in real-time adds a fourth dimension to the data collected, which is in the temporal domain [74]. After data acquisition, custom-written software was used to process and arrange each spectrum acquired in a scan to the spatial position from which it was acquired on the 2-D end of the fiber bundle.

5.4 Spatial calibrations of 2-D to 1-D fiber bundle

5.4.1 Spatial calibration on 1-D end

The spatial calibration was conducted on the 1-D end of the fiber bundle after it was aligned and fixed in place. The detector camera has a sensor array of 1002 rows (y -axis, spectral) and 1004 columns (x -axis, spatial), and it captures a 2-D spectral-spatial data (Fig. 5.4) from the fiberlets in 1-D end of the fiber bundle. Each coloured vertical line in Fig. 5.4 came from the light exiting the core of each fiberlet, which was then spectrally dispersed along the y -axis of the sensor array. The y -axis was later converted to the calibrated spectral bands. The position of each coloured vertical lines along the x -axis indicated the pixel columns used to image each fiberlet. Spectral information from each fiberlet would be acquired from the corresponding pixel columns during data processing. It can be observed from Fig. 5.4 that all the 1004 pixel columns of the sensor array were used to image the 100 fiberlets on the 1-D end. About 10 x -pixels were used to image each fiberlet. The dark line in Fig. 5.4 indicates the position of Fiberlet 4, which was damaged and thus inactive.

Fig. 5.4: Reference image taken by detector camera.

5.4.2 Spatial calibration on 2-D end

The second spatial calibration was done on the 2-D end of the fiber bundle, imaging an area of about $1.11 \times 1.32 \text{ mm}^2$. Fig. 5.5(a) shows the photograph of all the fiberlets on the 2-D end-face which were illuminated from the other end of the fiber bundle. Using Fig. 5.5(a), a digital mask of the 2-D end-face was created as shown in Fig. 5.5(b), containing the position and numbering of some fiberlets. The numbers on some of the fiberlets in Fig. 5.5 indicate their numbering. The white triangle in Fig. 5.5(a) indicates the position of Fiberlet 4 which was inactive and thus appeared to be dark. The spectrum acquired from each fiberlet in Fig. 5.4 will eventually be placed in the corresponding position in Fig. 5.5(b). The packing ratio of the fiberlets' cores on the 2-D end-face is about 55% [Fig. 5.5(b)].

Fig. 5.5: (a) Photograph and (b) digital mask of fiberlets on 2-D end-face.

5.5 Preparation of bio- and phantom tissue samples

In the reflectance imaging of phantom tissue sample, the sample used was a simulated tissue (Simulab Corporation) placed on a glass slide. This phantom tissue is a standard test sample used for proof of concept studies. A black tape was then placed on the tissue phantom. In the reflectance imaging of bio-sample, chicken breast tissue devoid of fat and

skin was used, with a visible blood clot on the surface. This part of the chicken breast was chosen so that the blood clot could provide a contrast in the image. The bio-sample was placed on the glass slide. A 99% reflectance standard (SRS-99-010, Labsphere) was also imaged as White data for reflectance imaging.

In fluorescence imaging, fluorescent powder (Ultra-orange/yellow fluorescent power, Medtech Forensics) was coated on the tissue phantom to simulate the different stages of tumour. All the imaged objects were kept at a distance of about 0.5 mm away from the 2-D end-face of the fiber bundle. The samples were manually moved using high-resolution mechanical translational stages during imaging.

5.6 Data acquisition

Data acquisition was done using the dedicated software of the detector camera (SOLIS, Andor). The selected region was 1004×756 pixel² (spatial $\times$ spectral), corresponding to the spectral range of interest from 400 nm - 1000 nm. Although the exposure time was set to 0.1 s, the software set the kinetic cycle time to 0.16221 s. Therefore the images were acquired at a rate of about 6.16 Hz. The electron-multiplying gain of the detector camera was turned off for reflectance imaging, but set to 100 for fluorescence imaging. During the experiment, the detector camera captured a series of 1004×756 pixel² images at a rate of about 6.16 Hz until the number of images taken matched the pre-determined number of images to capture. The images were named in sequence and saved as separate files after the experiment.

5.7 Data processing and visualization

Data processing was done offline using MATLAB®. In transmittance imaging, Sample data were acquired from the bars of the United States Air Force (USAF), and corrected using dark reference (Dark) and white reference (White) using Eq. (5.1) to get the

Transmittance data. Dark data were acquired when the broadband light source was turned off and the forelens covered. It represents the image of dark current noise where the transmittance was 0%. White data were acquired by imaging a clear region of the USAF chart where the transmittance was taken to be 100%. A set of ten images were taken and averaged to give the Dark and White data. x and λ refer to the column and calibrated spectral band allocated to the row of the sensor array's selected region, respectively. Frame refers to the image sequence taken for Sample data. Smooth is the 9-point moving average in the spectral direction for spectrum smoothing.

$$\text{Transmittance}(x, \lambda, \text{Frame}) = \text{Smooth} \left[\frac{\text{Sample}(x, \lambda, \text{Frame}) - \text{Dark}(x, \lambda)}{\text{White}(x, \lambda) - \text{Dark}(x, \lambda)} \right]. \quad (5.1)$$

In reflectance imaging, Sample data were acquired from the sample, and corrected using dark reference (Dark) and white reference (White) using Eq. (5.2) to get the Reflectance data. 80 images were captured. Dark data were acquired when the broadband light source was turned off and the forelens covered. It represents the image of dark current noise where the reflectance was 0%. White data were acquired by imaging the 99% reflectance standard where the reflectance was 99%. A set of ten images were taken and averaged to give the Dark and White data.

$$\text{Reflectance}(x, \lambda, \text{Frame}) = \text{Smooth} \left[\frac{\text{Sample}(x, \lambda, \text{Frame}) - \text{Dark}(x, \lambda)}{\text{White}(x, \lambda) - \text{Dark}(x, \lambda)} \right] \times 0.99. \quad (5.2)$$

In fluorescence imaging, Sample data were acquired from the sample and 160 images were taken. The Sample data were corrected using a dark reference (Dark) and the quantum efficiency of the detector camera (QE) using Eq. (5.3) to get the Fluorescence data. Dark data were acquired when the laser was turned off and the forelens covered. It represents the image with dark current noise without any fluorescence. QE took into account the varying

sensitivities the detector camera had with different wavelengths. A set of ten images were taken and averaged to give the Dark data. Norm was to normalise the entire data set to one.

$$\text{Fluorescence}(x, \lambda, \text{Frame}) = \text{Norm} \left\{ \frac{\text{Smooth}[\text{Sample}(x, \lambda, \text{Frame}) - \text{Dark}(x, \lambda)]}{\text{QE}(\lambda)} \right\}. \quad (5.3)$$

The processed data had a spatial-spectral-frame data of $1004 \times 756 \times \text{Frame}$. Using the spatial calibration done on the 1-D end of the fiber bundle, the spectrum for each fiberlet was extracted from the relevant spatial positions to form a fiberlet-spectral-frame data of $100 \times 756 \times \text{Frame}$. Since Fiberlet 4 was inactive, its spectrum was assigned to be zero. Using the data from each frame, a digital reconstruction step was done to remap the spectrum of each fiberlet back to the respective position on the 2-D end of the fiber bundle. In order to get a correct visualization of the imaged sample, the data were flipped horizontally in the spatial direction as the left side of the 2-D end of the fiber bundle was used to image the right side of the sample, and *vice versa*.

5.8 Results and discussion

The results of the HS measurements using the USAF resolution chart to determine the lateral resolution of the system are shown in this section. The HS results from bio- and fluorescent phantom tissue samples representing different stages of cancer growth using reflectance and fluorescence imaging modalities are also included.

5.8.1 Lateral resolution

The USAF chart was imaged in transmittance imaging instead of reflectance imaging, as it could not be properly imaged by reflectance imaging. Using the current configuration as shown in Fig. 5.1 (reflectance imaging), light is incident on surface of the USAF chart at an angle. As the USAF chart is optically-smooth, diffuse reflection would not occur for the light bouncing off its surface. Instead, the light would have been reflected at the same angle

away from the distal end-face of the fiber bundle and not be captured by the fiber bundle. Therefore transmittance imaging was used to determine the lateral resolutions of the system along the horizontal and vertical directions, even though the system was meant to illuminate and image sample from the same side for practical clinical applications.

Different Groups and Elements of the USAF chart were imaged to determine the lateral resolution of the system along the horizontal and vertical directions. The lateral resolutions along the horizontal and vertical directions were determined by imaging Group 1 Element 5 (G1E5) and G2E3 of the USAF chart, respectively. During imaging, the USAF chart was moving towards the left using a mechanical stage. The imaged regions are shown in Fig. 5.6. The test patterns on the USAF chart are opaque and therefore they have low transmittance and will appear to be dark in the datacube.

Fig. 5.6: Imaged regions of USAF chart (a) G1E5 and (b) G2E3.

Fig. 5.7 shows the transmittance mappings of nine datacubes at 500 nm, taken from G1E5 of the USAF chart. The features in the transmittance mappings shown in Fig. 5.7 can be matched to the test patterns shown in Fig. 5.6(a). The probe was initially imaging the vertical bars of G1E5 of the USAF chart. It can be seen from Fig. 5.7 that the targeted element of the USAF chart is moving towards the left during data acquisition with respect to image acquisition time. In the process, the vertical bars exit the image and the horizontal rows appear to be entering. This continues until the number “5” enters the image and data acquisition stops. By taking a closer look at frame 17 (top-right image of Fig. 5.7), it can be observed that the vertical lines of G1E5 are still distinguishable. The lateral resolution of the

system along the horizontal direction is determined using G1E5 of the USAF chart and is about 157.49 μm .

Fig. 5.7: Transmittance mappings of nine datacubes of G1E5 at 500 nm.

Fig. 5.8 shows the transmittance mappings of nine datacubes at 500 nm, taken from G2E3 of the USAF chart. The features in the transmittance mappings shown in Fig. 5.8 can be matched to the test patterns shown in Fig. 5.6 (b). The probe was initially imaging the number “3” on the left of the horizontal rows of G2E3 of the USAF chart. However, this feature was too small for it to be properly imaged and thus it appears as a group of about five yellow fiberlets in the transmittance mappings. It can be seen from Fig. 5.8 that the targeted element of the USAF chart is moving towards the left during data acquisition with

respect to image acquisition time. In this process, the horizontal rows of G2E3 enter the image and this is followed by a group of fiberlets with higher transmittance than the background. This group of fiberlets was imaging the vertical bars of G2E3 but they were too small to be properly imaged, therefore no distinct feature is seen. By taking a closer look at frame 30 (middle-right image of Fig. 5.8), it can be observed that the horizontal lines of G2E3 can still be distinguished. The lateral resolution along the vertical direction of the system is determined using G2E3 and is about $99.21 \mu\text{m}$.

Fig. 5.8: Transmittance mappings of nine datacubes of G2E3 at 500 nm.

5.8.2 Reflectance imaging of phantom tissue sample

The optical reflectance between normal tissue and tumour are different [20]. A black tape was used to simulate the region of unhealthy tissue having different reflectivity values. The sample is shown in Fig. 5.9 and was imaged while being illuminated by two flexible light guides to deliver light from a broadband white light source. The sample was divided into Regions R1 and R2. Region R1 is the phantom tissue sample representing normal tissue, while Region R2 is the black tape representing a tumour located on the tissue surface with different reflectance properties [18,20]. The sample was manually moved using a mechanical stage towards the right of the 2-D end of the fiber bundle during data acquisition. The image of the fiber bundle in Fig. 5.9(b) shows its initial position and the arrow indicates it was moving towards the left with respect to the sample during data acquisition. 80 frames were taken at a rate of about 6.16 Hz, in about 12.81s.

Fig. 5.9: (a) Simulated phantom tissue sample and (b) photograph of the 2-D end of fiber bundle superimposed on the imaged region of sample.

Each frame captured by the detector camera of the snapshot HS video-endoscope was used to build a 3-D datacube. Three cut-datacubes are shown in Fig. 5.10. These 3-D datacubes show the 4-D data (spatial-spatial-spectral-temporal) captured using the snapshot HS video-endoscope. It can be observed that with respect to the sample, the 2-D end of the fiber bundle was moving towards the left during data acquisition.

Fig. 5.10: Cut-datacubes acquired using frames (a) 21, (b) 35 and (c) 44.

Fig. 5.11 shows the reflectance mappings of nine wavelengths and datacubes. By looking at the frames in Fig. 5.10 and Fig. 5.11 sequentially, it can be observed that the proposed system was able to perform HS reflectance imaging in a snapshot configuration. The different reflectance between Regions R1 and R2 was captured and they can be easily differentiated from each another. The 2-D end of the fiber bundle was initially imaging Region R1 of high reflectance, representing normal tissue region. Then it moved to the left with respect to the sample and entered Region R2 of low reflectance, representing the abnormal region of the tissue. The sharp tip on the right of Region R2 was clearly imaged. This continued in the same direction while imaging Region R2 till the data acquisition stopped. These depict the actual relative motion between them during data acquisition as shown in Fig. 5.9(b). The shape of Region R2 is also correctly represented in the experimental results.

Fig. 5.11: 4-D reflectance mappings of nine selected wavelengths and datacubes.

The mean reflectance spectra and standard deviations of Regions R1 and R2 are shown in Fig. 5.12. Each data set was calculated from 27 spectra. The spectra of Region R1 were acquired from 9 fiberlets whose positions are indicated by the red arrow box in the intensity mapping shown for 500 nm in Fig. 5.11 and from Frame 3-5. The spectra of Region R2 were acquired from 9 fiberlets whose positions are indicated by the yellow arrow box in the intensity mapping for 700 nm as shown in Fig. 5.11 and from Frame 35-37.

Fig. 5.12: Mean reflectance spectra with standard deviations of Regions R1 and R2.

The spectra in Fig. 5.12 show that the 4-D HSI probe could capture the detailed reflectance spectra of Regions R1 and R2 while there was a relative motion between the sample and the 2-D end of the fiber bundle. It can be observed that Region R1 (phantom tissue sample) had a much higher reflectance than Region R2 (black tape) along the entire spectral range of interest. The average standard deviations of the reflectance spectra of Regions R1 and R2 are about $\pm 2.49\%$ and $\pm 0.42\%$, respectively.

The results in this section show that the 4-D HSI probe was able to capture the HS reflectance of different parts of the imaged sample throughout the duration of data acquisition. Reflectance intensity mappings of appropriate wavelengths can be selected to spectrally distinguish one region from another for diagnostic applications. The spectral information collected from known samples can be stored in a data library and used for identification and quantification.

5.8.3 Reflectance imaging of bio-sample

A chicken breast tissue with a blood clot was used as the bio-sample (Fig. 5.13). The images in Fig. 5.13 were acquired from the same sample but appear to have different colours due to the different illuminations and cameras used. The sample was divided into

Regions B1, B2 and B3. Region B1 was the chicken breast tissue. Region B2 was a thin layer of blood clot on the chicken breast tissue. It can be observed from Fig. 5.13(b) that the chicken breast tissue was still partially visible in Region B2. Region B3 was the blood clot. The sample was manually moved upwards using a mechanical stage during data acquisition. The image of the fiber bundle in Fig. 5.13(b) shows its initial position and the arrow indicates it was moving downwards with respect to the sample. 80 frames were taken at a rate of about 6.16 Hz, in about 12.81s.

Fig. 5.13: (a) Bio-sample and (b) photograph of the 2-D end of fiber bundle superimposed on sample.

Fig. 5.14 shows the reflectance mappings of nine datacubes at 600 nm. By looking at the frames in Fig. 5.14 sequentially, it can be further confirmed that the proposed system was able to perform HS reflectance imaging in a snapshot configuration. The different reflectance between Regions B1, B2 and B3 can be differentiated from each another. The 2-D end of the fiber bundle was initially imaging Region B1 of high reflectance. Then it moved downwards with respect to the sample and started to image Region B2 of moderate reflectance on its left. Following this path, it started to image Region B3 of low reflectance and proceeded to image Region B1 again before data acquisition stopped. These depict the actual relative motion between them during data acquisition as in Fig. 5.13(b). There was a small area of Region B1 in between Regions B2 and B3 near the centre of the blood clot. This area is seen in Frame 33 of Fig. 5.14 which correctly represents its shape and size.

Fig. 5.14: Reflectance mappings of nine datacubes at 600 nm.

The mean reflectance spectra and standard deviations of Regions B1, B2 and B3 are shown in Fig. 5.15. Each data set was calculated from 27 spectra. The spectra of Regions B1, B2 and B3 were acquired from 9 fiberlets whose positions are indicated by the arrow boxes in Fig. 5.14, and from Frames 1-3, 26-28 and 53-55, respectively. The spectra in Fig. 5.15 show that the 4-D HSI probe could capture the detailed reflectance spectra of Regions B1, B2 and B3 while there was a relative motion between the sample and the 2-D end of the fiber bundle. It can be observed that Region B1 (chicken breast tissue) had the highest reflectance, while Region B3 (blood clot) had the lowest. The reflectance spectrum of Region B2 is in between the spectra of Regions B1 and B3. This could be due to Region B2

having the thin layer of blood clot while the chicken breast tissue underneath it was still partially visible. The average standard deviations of the reflectance spectra of Regions B1, B2 and B3 are about $\pm 1.31\%$, $\pm 1.37\%$ and $\pm 0.98\%$, respectively.

Fig. 5.15: Mean reflectance spectra with standard deviations of Regions B1, B2 and B3.

5.8.4 Fluorescence imaging of phantom tissue sample

A phantom tissue sample with applications of fluorescent powder (Fig. 5.16) was used to simulate different stages of cancer growth in colon. The images in Fig. 5.16 were acquired from the same sample but appear to have different colours due to the different illuminations and cameras used. The sample was imaged while being illuminated by two flexible light guides delivering light from a 532-nm laser during the experiment. The fluorescent powder has emission wavelengths from about 500 nm - 700 nm, and falls within the emission maxima of biological endogenous fluorophores (280 nm - 690 nm) [125].

The sample was divided into Regions F1, F2 and F3. Region F1 has a thick layer of fluorescent powder (higher concentration) representing normal tissue having normal autofluorescence intensity. Region F2 has a thin layer of fluorescent powder (lower concentration) representing tumour growth in the intermediate stage with reduced

autofluorescence intensity. Region F3 is the simulated phantom tissue sample representing tumour growth in the advanced stage with very weak autofluorescence. The sample was manually moved upwards using a mechanical stage, then towards the left of the 2-D end of the fiber bundle during data acquisition. The fiber bundle in Fig. 5.16(b) shows its initial position and the arrows indicates it moving downwards then towards the right with respect to the sample during data acquisition. 160 frames were taken at a rate of about 6.16 Hz, in about 25.79 s.

Fig. 5.16: (a) Simulated phantom tissue sample and (b) photograph of the 2-D end of fiber bundle superimposed on sample.

Each frame was used to build a 3-D datacube. Although the full spectral range of 400 nm - 1000 nm was captured, only the data from 570 nm - 600 nm is used to build the cut-datacubes shown in Fig. 5.17. Fig. 5.17(a) shows Region F1 representing normal tissue with detection of high fluorescence intensity. Fig. 5.17(b) shows Region F3 representing tumour growth in the advanced stage with detection of very low fluorescence intensity. Fig. 5.17(c) shows Region F2 representing tumour growth in the intermediate stage with detection of low fluorescence intensity. These 3-D datacubes show the 4-D data (spatial-spatial-spectral-temporal) captured using the snapshot HS video-endoscope.

Fig. 5.17: Cut-databucubes acquired using frames (a) 18, (b) 58 and (c) 128.

Fig. 5.18 shows the fluorescence mappings of nine databucubes at 585 nm. By looking at the frames in Fig. 5.18 sequentially, it can be observed that the proposed snapshot system can be used to perform HS fluorescence imaging. The fluorescence intensities of Regions F1, F2 and F3 were captured and the information can be used to differentiate one from another. The 2-D end of the fiber bundle was initially imaging Region F3 of very low fluorescence intensity. It moved downwards with respect to the sample and entered Region F1 of high fluorescence intensity. Then it entered Region F3 before moving towards the right with respect to the sample and entered Region F2 of low fluorescence intensity. It continued in this path until it entered Region F3 before data acquisition stopped. These depict the actual relative motion between them during data acquisition as shown in Fig. 5.16(b). It is to be noted that there was uneven illumination on the phantom sample where the illumination on the left was stronger. The shapes of Regions F1 and F2 are also correctly represented in the experimental results.

The mean fluorescence spectra and standard deviations of Regions F1, F2 and F3 are shown in Fig. 5.19. Each data set was calculated from 27 spectra and normalised to the

maximum value of the mean fluorescence spectrum of Region F1. The spectra were acquired from the same 9 fiberlets whose positions are indicated by the green and red arrow boxes in Fig. 5.18. For the purpose of comparing the spectral intensity of Regions F1, F2 and F3, the effect of uneven illumination on the sample is reduced by acquiring the spectra from the same fiberlets. The spectra of Regions F1, F2 and F3 were acquired from Frame 17-19, 127-129 and 57-59, respectively.

Fig. 5.18: Fluorescence mappings of nine datacubes at 585 nm.

Fig. 5.19: Mean fluorescence spectra with standard deviations of Regions F1, F2 and F3.

The spectra in Fig. 5.19 illustrate that the 4-D HSI probe could capture the detailed fluorescence spectra of Regions F1, F2 and F3 while there was a relative motion between the sample and the 2-D end of the fiber bundle. The peak fluorescence wavelength was about 585 nm. It can be observed that strong fluorescence was detected from Region F1 (thick fluorescent powder region with higher concentration) representing normal tissue with normal autofluorescence intensity. A relatively weaker fluorescence was detected from Region F2 (thin fluorescent powder region with lower concentration) representing tumour growth in the intermediate stage with reduced autofluorescence intensity. A very weak fluorescence was detected from Region F3 (phantom tissue sample) representing tumour growth in the advanced stage with very weak autofluorescence. The average standard deviations of the fluorescence spectra of Regions F1, F2 and F3 from 500 nm - 700 nm are about ± 0.0368 , ± 0.0213 and ± 0.0026 , respectively.

The results in this section show that the 4-D HSI probe captured the HS fluorescence of different parts of the imaged region throughout the duration of data acquisition. The system captures fluorescence spectra to reveal the type and concentration of fluorophores in the samples, which can lead to tumour staging and other related disease diagnosis applications.

5.9 Summary

A 2-D to 1-D fiber bundle has been custom-fabricated which converts the pushbroom HS imager into a snapshot configuration. The fiber bundle is flexible and has a small distal end, enabling it to be used as an imaging probe that can be inserted into the colon for minimally invasive and *in vivo* investigations for the detection of cancer. By acquiring data frames continuously, these factors come together to form a snapshot HS video-endoscope for endoscopic colon imaging.

The detailed instrumentation scheme of the proposed system has been proposed and its feasibility demonstrated. The USAF chart was imaged in transmittance imaging and the lateral resolutions of the system along the horizontal and vertical directions were found to be 157.49 μm and 99.21 μm , respectively. Reflectance and fluorescence imaging were conducted when the light source and the probe were both on the same side as that of the imaged samples. This is the expected configuration during *in vivo* imaging of the internal body cavity. It is also to be noted that the probe can be integrated with a control and locomotion option as in conventional endoscopes to avoid the need for sample movement when this is used inside body cavities.

A frame rate of about 6.16 Hz was attained, and each frame was converted into a 3-D datacube with 756 spectral bands. The 3-D datacubes and intensity mappings can provide vast amount of information, which includes the spatial features (shape and size), spectral signatures (756 bands), speed and direction of the imaged samples. The spectral information can also be seen in the line plots. These promising results confirm the successful implementation of such a 2-D to 1-D fiber bundle serving its use as a snapshot HS video-endoscopic probe. The snapshot HS video-endoscope illustrated in this chapter used the

flexible 2-D to 1-D fiber bundle for potential bio-imaging applications for the first time. It also captures 756 spectral bands which are significantly more than existing snapshot HS video-endoscopes which can generally capture only about 50 spectral bands. With more spectral bands available, limitations such as a reduced spectral range, insensitivity to certain narrow spectral band and inability to capture detailed spectral signatures, can be avoided.

The use of such a HS video-endoscope with a flexible 2-D to 1-D fiber bundle can be a potential alternative to conventional fiber-optic imaging systems. The information collected by a HS video-endoscope has an additional spectral dimension of the order of several hundred wavelength bands. Conventional video-endoscope using colour camera gives only little spectral information from three bands. In this aspect, HS video-endoscopes can be especially useful when detailed spectrum is required for classification and quantification to give functional information such as haemoglobin saturation [66]. It is also valuable in cases where multiple excitation sources are used to excite multiple fluorescent tags and the HS data can be used to differentiate the fluorescent tags even when the excitation and emission spectra are over-lapping but distinct [60]. Currently, many conventional setups in the field of optogenetics and neuronal imaging can only image one fluorescent tag in each frame [134-136]. Such studies can benefit by using HS video-endoscope with a flexible and compact distal end. It can be used in more complex and non-invasive studies to capture detailed spectral information from multiple fluorescent tags.

A future improvement to the probe system is to use smaller fiberlets so that more can be packed within the fiber bundle. The current system images 100 fiberlets on the 1-D end using all the 1004 pixel columns. While the maximum number of fiberlets that can be effectively imaged by the snapshot imager is 1004, which is the number of pixel column of

the sensor array. By using smaller fiberlets, more can be packed along the 1-D end. Spectral information from more spatial points will be collected and data collection by the sensor array becomes more efficient. The spatial resolution of the image is also expected to be better. Another possible improvement to the system is to increase its frame rate from the current about 6.16 Hz to 20 Hz so that it becomes real-time. The current frame rate is limited by the detector camera and exposure time. This can be made a complete real-time system by replacing the detector camera with another one having faster readout rate and by using a lower exposure time.

The ensuing chapter illustrates a HS photoacoustic spectroscopy system to directly measure the normalised optical absorption coefficient of highly-absorbing samples. The system uses an optical absorption coefficient reference to remove the need to perform spectral calibrations to account for the wavelength-dependent transmittance and reflectance of the optical components used in the system.

Chapter 6: Hyperspectral photoacoustic spectroscopy of highly-absorbing bio-samples

Photoacoustic spectroscopy has been used to measure optical absorption coefficient and the application of tens of wavelength bands in photoacoustic spectroscopy has been reported. Using optical methods, absorption-related information is generally derived from reflectance or transmittance values. Hence measurement accuracy is limited for highly-absorbing samples where the reflectance or transmittance can be too low to give reasonable signal-to-noise ratio. In this context, this chapter proposes and illustrates a hyperspectral photoacoustic spectroscopy system to directly measure the normalised optical absorption coefficient of highly-absorbing samples. Measurements are carried out for 461 wavelength bands and the use of an optical absorption coefficient reference removes the need to perform spectral calibration to account for the wavelength-dependent transmittance and reflectance of the optical components. The normalised optical absorption coefficient spectrum of the highly-absorbing iris is acquired. The proposed concepts and the feasibility of the developed diagnostic medical imaging system are demonstrated by using fluorescent microsphere suspensions and porcine eyes as test samples.

6.1 Introduction

Uveal melanoma is a type of intraocular cancer which can occur in the iris and if untreated, can lead to blindness and deaths [15,137]. Characterisation of different conditions of the eyes can be used to diagnose the type and condition of diseases. This has been demonstrated in the functional imaging of the ocular micro-circulation by measuring the oxygen saturation in the radial iris arteries using photoacoustic (PA) imaging [138].

PA imaging can be used to detect uveal melanoma and its spread along the depth of the iris, by investigating the time of arrival of the detected PA signals. Hence, due to the deep imaging capability of PA imaging [91], it can still potentially detect uveal melanoma even if the diseased site is located beneath healthy iris. Conventional optical imaging in reflection mode would be more suitable to determine the lateral size of uveal melanoma when it

occurs on the surface of the iris [15]. It lacks depth-related information and the depth of the diseased site cannot be determined. Another benefit of using PA imaging is that it has the potential to form a hybrid-modality ocular imaging system by integrating it with ultrasound imaging. The optical absorption-based information that is available through PA imaging and structural information available through ultrasound imaging are integrated to provide complementary information for better diagnosis.

Using PA instead of optical methods to measure the optical absorption coefficient (OAC) of the highly-absorbing iris is beneficial. PA measurement is a direct measurement of OAC itself and it gives an enhanced detection limit and dynamic range [88]. PA techniques had been used in imaging [139,140] and measurement of OAC [88,105,141-143], thermal diffusivity [142] and Grüneisen parameter [89,106]. In most of these cases, only one or few excitation wavelengths were used in the measurement [88,89,141-143], though some of the reported cases used few tens of wavelength bands [105,106,140]. However, there is a need for higher measurement accuracy and better spectral details.

The common method to measure fluence in many PA setups is to use a beam sampler/splitter to direct part of the excitation source to an energy sensor, such as a photodiode. The transmittance and reflectance of the optical components along the light path between the photodiode and the sample is wavelength-dependent. When this is not accounted, the photodiode cannot correctly measure the relative fluence applied to the sample at different wavelengths. It has been used to correct the pulse-to-pulse energy fluctuations but this does not account for the wavelength-dependent transmittance and reflectance of these optical components. For accurate measurement, calibration is required to adjust the energy measurement to give the actual relative fluence applied to the sample.

Generally for a simple setup with a short spectral range and few optical components, it is assumed that the optical components between the photodiode and sample have wavelength-independent transmittance and reflectance. However, this does not apply in setups using multiple optical components between the photodiode and sample across a broad wavelength range, covering the visible and near-infrared wavelength band.

In this context, this chapter presents and demonstrates a novel concept based on hyperspectral photoacoustic spectroscopy (HS-PAS) to acquire the normalised OAC spectrum of highly-absorbing bio-samples. This allows the OAC characterisation of healthy iris and uveal melanoma in the iris using PA method, which can be used to detect diseases. Such characterisation is important to determine the optimal wavelength for PA excitation such that there is good contrast difference between healthy iris and uveal melanoma. More wavelength bands for interrogation within a spectral band enable OAC characterisation with detailed spectral signatures, higher spectral precision and resolution. Enucleated porcine eyes were used as the test samples. The use of an OAC reference is also proposed to serve as a reference whose PA measurement is compared with that of the sample. This removes the need to perform spectral calibration to account for the wavelength-dependent transmittance and reflectance of the optical components. Optical components can also be added or removed from the setup without performing another spectral calibration.

6.2 Theory

The basic equation governing PA measurements is shown in Eq. (2.4) [31,93,104]:

$$P_0(\text{Temp}, \lambda) = \Gamma(\text{Temp})F(\lambda)\mu(\lambda), \quad (2.4)$$

where P_0 is the initial pressure rise of the PA wave, Γ is the dimensionless Grüneisen parameter, F is the optical fluence, μ is the OAC, Temp is the temperature in medium and λ

is the optical excitation wavelength. It is also mentioned in Sec. 2.3.4 that it is cumbersome to determine the actual values of P_0 , Γ and F to calculate the actual value of μ from the experimental point of view. In many cases, P_0 and F are measured in arbitrary values.

P_V is the maximum amplitude of the ultrasound transducer (UST) signal after Hilbert transformation and it is an indication of the strength of P_0 . Hilbert transformation is widely used in analytical signal analysis to pick up the envelopes of vibration signals [107].

$$P_V(\text{Temp}, \lambda) = \text{Max}\{\text{Hilbert}[P_{\text{UST,raw}}(\text{Temp}, \lambda)]\}, \quad (6.1)$$

where $P_{\text{UST,raw}}$ is the raw signals from the UST.

F_V is the area under the raw signals from the photodiode ($F_{\text{PD,raw}}$) and is a measure of the excitation fluence.

$$F_V(\lambda) = \text{Sum}[F_{\text{PD,raw}}(\lambda)]. \quad (6.2)$$

The photodiode's responsivity Resp has to be taken into account for an accurate measurement of the fluence ratio of different wavelengths. By taking into account the photodiode's responsivity and integrating Eq. (6.1) and Eq. (6.2) into Eq. (2.4), it becomes

$$P_V(\text{Temp}, \lambda) \propto \Gamma(\text{Temp}) \frac{F_V(\lambda)}{\text{Resp}(\lambda)} \mu(\lambda). \quad (6.3)$$

Two sets of PA measurements are required to calculate the normalised OAC spectrum of the sample. The first set is from the sample while the second is from the OAC reference. The PA signals from the sample are compared with that from the OAC reference which can be expressed as Eq. (6.4), derived from Eq. (6.3). Equation (6.4) is re-written as Eq. (6.5). Two functions (Norm and Smooth) are applied to Eq. (6.5) to acquire the normalised OAC spectrum of the sample ($\mu_{\text{Ref_N}}$) as shown in Eq. (6.6). The function Norm is a division of the spectrum by its maximum value, and Smooth is an 11-point moving average. The experimental data ($P_{V,\text{Ref}}$, $P_{V,\text{Ref}}$, $F_{V,\text{Ref}}$ and $F_{V,\text{Sam}}$) and the normalised OAC spectrum of the

reference ($\mu_{\text{Ref_N}}$) are required in Eq. (6.6) to obtain the sample's normalised OAC spectrum ($\mu_{\text{Sam_N}}$).

$$\frac{P_{V,\text{Sam}}(\text{Temp},\lambda)}{P_{V,\text{Ref}}(\text{Temp},\lambda)} = \frac{\Gamma_{\text{Sam}}(\text{Temp}) \frac{F_{V,\text{Sam}}(\lambda)}{\text{Resp}(\lambda)} \mu_{\text{Sam}}(\lambda)}{\Gamma_{\text{Ref}}(\text{Temp}) \frac{F_{V,\text{Ref}}(\lambda)}{\text{Resp}(\lambda)} \mu_{\text{Ref}}(\lambda)}, \quad (6.4)$$

$$\mu_{\text{Sam}}(\text{Temp},\lambda) = \frac{\Gamma_{\text{Ref}}(\text{Temp})}{\Gamma_{\text{Sam}}(\text{Temp})} \frac{F_{V,\text{Ref}}(\lambda) P_{V,\text{Sam}}(\text{Temp},\lambda)}{F_{V,\text{Sam}}(\lambda) P_{V,\text{Ref}}(\text{Temp},\lambda)} \mu_{\text{Ref}}(\lambda), \quad (6.5)$$

$$\mu_{\text{Sam_N}}(\lambda) = \text{Norm} \left\{ \text{Smooth} \left[\frac{F_{V,\text{Ref}}(\lambda) P_{V,\text{Sam}}(\lambda)}{F_{V,\text{Sam}}(\lambda) P_{V,\text{Ref}}(\lambda)} \mu_{\text{Ref_N}}(\lambda) \right] \right\}. \quad (6.6)$$

It is to be mentioned that Temp is assumed to be constant during the measurements. Therefore $\Gamma_{\text{Ref}}(\text{Temp})$, $\Gamma_{\text{Sam}}(\text{Temp})$ and the variable Temp do not appear in Eq. (6.6) which involves normalization. Due to fluctuation of the laser energy even under the same laser setting, the ratio of $\frac{F_{V,\text{Ref}}(\lambda)}{F_{V,\text{Sam}}(\lambda)}$ is not always one. This ratio is used to account for such deviation in Eq. (6.6). In short, using the OAC reference removes the need to perform spectral calibration to account for the wavelength-dependent transmittance and reflectance of the optical components.

6.3 Instrumentation of HS-PAS

For ocular measurement, both the eye and the OAC reference use the configuration shown in Fig. 6.1(a). The configuration in Fig. 6.1(b) is used to validate the proposed concept to measure the normalised OAC spectrum of fluorescent microsphere suspensions as test samples. Although light passes through different media between objective lens 2 and the sample in the two configurations, it is assumed that the glass slide, water and air have flat transmittance spectra from 410 nm - 870 nm.

Fig. 6.1: Schematic diagrams of HS-PAS setup for (a) measurement with eye and OAC reference and (b) validation.

A tunable pulsed nanosecond laser (Vibrant 355 II, Opotek Inc.) provides optical excitation for PA measurement. The spectral range of interest is 410 nm - 870 nm with 1 nm spectral interval. 461 wavelength bands are used in each measurement, which far exceeds the number of wavelength used in hyperspectral measurement (about 100-200), as defined by Fresse *et al.* [48]. The laser has an optical parametric oscillator producing collinear Signal (410 nm - 710 nm) and Idler (710 nm - 2400 nm) beams. Under the same laser setting (energy and transmission), the pulse energy generally decreases with wavelength. A Glan-laser polariser separates the Signal and Idler beams. The polariser is first positioned for the emission of Signal beam from 410 nm - 710 nm. In order to use the 711 nm - 810 nm bands, the polariser has to be manually repositioned for the emission of Idler beam.

The laser fires 40 pulses at 10 Hz at each wavelength. When the pulse reaches the plate beam splitter (BSW10, Thorlabs), the pulse is partially reflected and directed towards the neutral density filter (ND30A, Thorlabs) and focused by objective lens 1 (UPlan FLN 10×, Olympus) onto the photodiode (SM05PD2B, Thorlabs). The pulse detected by the

photodiode triggers the digitizer (Razor CompuScope 1622, GaGe, 200 MS/s). The transmitted pulse through the plate beam splitter travels towards objective lens 2 (MPlan N 5×, Olympus), which is partially submerged in water as shown in Fig. 6.1(a). Light passes through a glass slide before reaching the sample held in place by a 3-axis motorised stage (T-LS28M, Zaber Technologies). Upon sample excitation, the PA wave produced is directed by the glass slide towards the UST (V110-RM, Olympus Panametrics-NDT, frequency 5.0 MHz, nominal element size 6 mm) for detection. A pre-amplifier (5662, Olympus Panametrics-NDT) with a 54 dB gain amplifies the detected signal before reaching the digitizer. When the digitizer is triggered, the signals from both the photodiode and UST are acquired. The signals are averaged over 40 pulses for each wavelength to improve the signal-to-noise ratio. Custom-developed LabVIEW® software (Appendix D) is used to control the laser, 3-axis motorised stage and digitizer, and to save the averaged signals.

6.4 Preparation of porcine eye sample

Randomly selected enucleated eye samples from porcine (*Sus scrofa domestica*) were acquired from local abattoir. Extraocular tissues were removed from the eye samples before being placed and transported on ice. This helps to keep the eye samples fresh until the experiments began. Visual inspections were conducted on the eye samples and only those found to be free of signs of deterioration were used for testing. The study was conducted within 6 hours after sample acquisition and followed Nanyang Technological University's biosafety regulations and regulations of Agri-Food & Veterinary Authority of Singapore.

6.5 Data processing

The data acquired from the sample and OAC reference are processed by custom-written script in MATLAB®. Hilbert transformation is applied to the UST signal and the peak

amplitude of the transformed signal (P_V) is acquired. The area under the photodiode signal is calculated to give F_V . UST and photodiode signals acquired from the OAC reference using excitation of 500 nm are shown in Fig. 6.2.

Fig. 6.2: (a) UST and (b) photodiode signals of OAC reference using 500-nm excitation.

By repeating the above for all wavelengths, $P_V(\lambda)$ and $F_V(\lambda)$ spectra are acquired. The $P_V(\lambda)$ and $F_V(\lambda)$ spectra of the OAC reference and a sample (Red fluorescent microsphere suspension) are shown in Fig. 6.3.

Fig. 6.3: (a) $P_V(\lambda)$ and (b) $F_V(\lambda)$ of the OAC reference and sample.

6.6 Results and discussion

The results first show the measured normalised OAC spectrum of the OAC reference, which is followed by the validation using fluorescent microsphere suspensions. Then the measured normalised OAC spectrum and multispectral PA imaging of the enucleated porcine eye sample is shown.

6.6.1 Normalised OAC spectrum of OAC reference

A grey tape was used as the OAC reference. Optical method was used to acquire the normalised OAC spectrum since its reflectance and transmittance were sufficiently high within 410 nm - 870 nm to give reasonable signal-to-noise ratio. Light attenuation through the OAC reference was assumed to comply with Beer-Lambert law, as stated in Eq. (6.7) [142].

$$T(\lambda) = [1 - R(\lambda)]\exp[-\mu_{\text{Ref}}(\lambda)L], \quad (6.7)$$

where T , R and L are the transmittance, reflectance and thickness of the OAC reference, respectively.

The assumed behaviour of light through the OAC reference is shown in Fig. 6.4(a), where I_0 is the incident intensity of light and I is the intensity as it travelled from the front to the back surface in x -direction. Equation (6.8), derived from Eq. (6.7) [142], calculates the normalised OAC spectrum of the reference. L is a constant and does not appear in Eq. (6.8) which involves normalization.

$$\mu_{\text{Ref}_N}(\lambda) = \text{Norm} \left\{ \text{Smooth} \left[-\ln \frac{T(\lambda)}{1-R(\lambda)} \right] \right\}. \quad (6.8)$$

A broadband source (MI-150, Edmund Optics) and two lenses (LB1761-A and LB1471-A, Thorlabs) were used to produce the collimated white light. The setup in Fig. 6.4(b) was used to measure $T(\lambda)$, where the OAC reference was placed before the integrating sphere (4P-GPS-060-SF, Labsphere). Part of the transmitted light travels through the optical fiber (QP400-1-VIS-NIR, Ocean Optics) and was detected by the spectrometer (USB4000, Ocean Optics). The SpectraSuite® software (Ocean Optics) calculated $T(\lambda)$ after taking into account the dark current of the spectrometer and 100% transmittance reference (blank sample). The setup in Fig. 6.4(c) measured $R(\lambda)$, where the reference was a 99% reflectance

standard (SRS-99-010, Labsphere). After measuring $T(\lambda)$ and $R(\lambda)$, they were applied in Eq. (6.8) to calculate $\mu_{\text{Ref}_N}(\lambda)$. $\mu_{\text{Ref}_N}(\lambda)$ is relatively flat ranging from 0.94-1, as shown in Fig. 6.5. $\mu_{\text{Ref}_N}(\lambda)$ and the experimental data [$P_{V,\text{Ref}}(\lambda)$, $P_{V,\text{Sam}}(\lambda)$, $F_{V,\text{Ref}}(\lambda)$ and $F_{V,\text{Sam}}(\lambda)$] from Sec. 6.5 were applied in Eq. (6.6) to get $\mu_{\text{Sam}_N}(\lambda)$.

Fig. 6.4: (a) Assumed behaviour of light in OAC reference, experimental setup to measure (b) transmittance and (c) reflectance of OAC reference.

Fig. 6.5: Normalised OAC spectrum of reference $\mu_{\text{Ref}_N}(\lambda)$.

6.6.2 Validation using fluorescent microsphere suspensions

The configuration in Fig. 6.1(b) was used to validate the proposed method to measure the normalised OAC spectrum, using Red, Crimson and Nile Red fluorescent microsphere suspensions (F8858, F8816 and F8825, respectively from Life Technologies) as test samples. Each suspension was placed in a cuvette and appeared to be opaque. The data acquired [$\mu_{\text{Ref}_N}(\lambda)$, $P_{V,\text{Ref}}(\lambda)$, $P_{V,\text{Sam}}(\lambda)$, $F_{V,\text{Ref}}(\lambda)$ and $F_{V,\text{Sam}}(\lambda)$] were incorporated in Eq. (6.6) to find the normalised OAC spectrum of the sample (Fig. 6.6).

Fig. 6.6: $\mu_{\text{Sam}_N}(\lambda)$ of Red fluorescent microsphere suspension.

Four measurements were taken for each fluorescent microsphere suspension and each set of results were averaged and normalised. Fig. 6.7 compares the acquired normalised OAC spectra of the three suspensions with their respective normalised absorption spectra (provided online by Life Technologies). There is dependence between the OAC and absorption spectra which are evident from Fig. 6.7. A rise in OAC will lead to a rise in absorption, and *vice versa*. This trend is clearly evident in all three suspensions, and it verifies that the proposed concept is capable of giving the characteristics of the OAC spectrum of highly-absorbing samples with 1-nm resolution. The peak wavelengths of the acquired normalised OAC spectrum and the given absorption spectrum (as per the specifications) differ from each other by 4 nm - 7 nm. These differences may be a result of the different setups used to acquire the OAC and absorption spectra. Using HS-PAS, the normalised OAC spectra from 410 nm - 870 nm of highly-absorbing samples were acquired with spectral resolution of 1 nm. Such a detailed normalised OAC spectrum allows the precise selection of suitable wavelengths for spectroscopic or multispectral imaging purposes. Also, using the proposed OAC reference removes the need to perform spectral calibrations to account for the wavelength-dependent transmittance and reflectance of the optical components between the photodiode and sample.

Fig. 6.7: Validation results using (a) Red, (b) Crimson and (c) Nile Red fluorescent microsphere suspensions.

6.6.3 Experiments using enucleated porcine eye samples

6.6.3.1 HS-PAS of iris of enucleated porcine eye sample

The configuration in Fig. 6.1(a) was used to acquire the normalised OAC spectrum of the iris of an enucleated porcine eye sample. The result in Fig. 6.8 shows the normalised OAC spectrum of the top surface of the iris.

Fig. 6.8: Measured normalised OAC spectrum of iris in porcine eye sample.

6.6.3.2 Multispectral PA imaging of enucleated porcine eye sample

In this study, three wavelengths of different normalised OAC of the iris were selected based on the results in Sec. 6.6.3.1. The selected wavelengths, 465 nm, 750 nm and 870 nm, had an OAC ratio of 1: 0.421: 0.183. The laser settings of these wavelengths were adjusted to have the same fluence. When a sample is excited by different wavelengths of the same fluence, the strength of the UST signal (P_V) is directly proportional to the OAC only. Therefore the P_V ratio using the selected wavelengths having the same fluence should be close to that of the measured normalised OAC of 1: 0.421: 0.183.

The laser transmissions of the selected wavelengths were adjusted to have the same fluence by using the OAC reference as the sample. The OAC reference had a relatively flat normalised OAC spectrum (Fig. 6.5). When the P_V of the three wavelengths were close to each other when the OAC reference was used, it implied that the fluence of these wavelengths were about the same. The laser transmissions for 465 nm, 750 nm and 870 nm were found to be 1%, 7% and 21%, respectively.

Each B-scan image was acquired using the earlier mentioned LabVIEW® software, which included the synchronization of the 3-axis stage to move 199 steps, each covering 80 μm . 20 pulses of the same wavelength were fired at each position. Each B-scan measurement took about 10 minutes to complete. The data were processed similarly as described in Sec. 6.5. Fig. 6.9(a) shows the schematic of the eye and Fig. 6.9(b-d) show the B-scan images at 465 nm, 750 nm and 870 nm, respectively.

Fig. 6.9: (a) Schematic of the eye, B-scan images across the centre of the eye using (b) 465 nm (c) 750 nm and (d) 870 nm.

Each B-scan image is made up of 2200×200 pixels² (z -depth $\times$ x -position). When compared with Fig. 6.9(a), B-scan images show features which are identified as the iris and posterior pole. The pigmented iris contains melanin and the posterior pole contains blood vessels, both melanin and blood are highly absorbing. Thus strong PA signals were acquired from these regions [14,144], but not from other parts like the optically clear cornea.

The strength of the PA signals from the top of the iris at different wavelengths of the same fluence was calculated by averaging the maximum amplitudes of 10 A-scans (P_V) within the white box in each B-scan image. The P_V ratio in the B-scan images of 465 nm,

750 nm and 870 nm was calculated to be 1: 0.457: 0.184. This ratio is very close to the ratio of 1: 0.421: 0.183 in the measured normalised OAC spectrum of the iris using HS-PAS. The similarity between these two ratios shows that the proposed concept and methodology are able to acquire fine spectral details (1-nm resolution) of the normalised OAC spectrum of the iris accurately.

A major limitation using conventional optical methods to measure the OAC of highly-absorbing iris is that the transmittance and reflectance signals may be too low to give accurate data for analysis (low signal-to-noise ratio). Although optical methods are non-destructive, fast and inexpensive [145], they do not enable direct measurement of absorption properties itself. Optical methods need to measure transmittance and reflectance values before absorption-based information is acquired. For this, common methods such as those based on Beer-Lambert law [142] and the theory of oblique-incidence reflectometry are employed [145-147]. Also, optical methods measuring transmittance need access to both the front and back of the iris, thus the eye cannot be left intact. When the sample is highly absorbing, using PA instead of conventional optical method for the OAC characterisation of the iris is more suitable. PA method is directly proportional to OAC and PA signals increase with OAC, providing an enhanced detection limit and improved dynamic range [88].

6.6.3.3 Adherence to guideline on exposure limit to laser radiation

For potential diagnostic clinical applications to characterise healthy and diseased sites in the iris for the detection of uveal melanoma, the exposure limit (EL) of the system is subjected to guidelines defined by International Commission on Non-Ionizing Radiation Protection for the skin [148]. For this purpose, where the illumination is targeted at the iris

and not the cornea and retina, the EL for skin is used as the guideline to protect the anterior parts of the eye [148].

The focal length of objective lens 2 (MPlan N 5×, Olympus), which was partially submerged in water during the measurement of eye sample, has to be calculated. The angular subtense θ is calculated using the numerical aperture (NA) of objective lens 2, which is 0.1, using Eq. (6.9) derived from Snell's law.

$$\theta = \sin^{-1} \frac{NA}{n_{\text{water}}} = \sin^{-1} \frac{0.1}{1.333} \approx 4.302^\circ, \quad (6.9)$$

where n_{water} is the refractive index of water. The front lens of objective lens 2 has a radius of 4.3 mm, and the focal length is calculated using Eq. (6.10) derived from Law of Sines.

$$\text{Focal length} = \frac{\text{Lens radius}}{\sin(\theta)} \sin(90 - \theta) = \frac{4.3}{\sin(\theta)} \sin(90 - \theta) \approx 57.16 \text{ mm}. \quad (6.10)$$

With a working distance of about 40 mm and the radius of the laser beam exiting objective lens 2 r_1 of about 2 mm (Fig. 6.10), the radius of the laser spot on the sample r_2 is calculated using Eq. (6.11).

$$r_2 = \frac{\text{Focal length} - \text{Working distance}}{\text{Focal length}} r_1 \approx 0.6003 \text{ mm}. \quad (6.11)$$

The area of the laser spot on the sample is therefore calculated to be about 1.132 mm^2 .

Fig. 6.10: Schematic of laser beam exiting objective lens 2.

Eight wavelengths within the spectral range of 410 nm - 870 nm were selected to represent all the wavelengths in the spectral range. The selected wavelengths, respective

laser transmission settings and the measured pulse energy (Nova Display and 12AV1, Ophir) are in Table 6.1.

Table 6.1: Selected wavelengths and measured pulse energy.

λ (nm)	Laser transmission setting (%)	Pulse energy (μJ)
410	1	2.254
475	1	3.050
541	2	4.909
620	2	4.350
700	4	2.721
740	7	3.417
800	7	5.669
870	23	4.994

The detailed calculations and relevant analysis on the adherence to guideline on exposure limit to laser radiation for different wavelengths are given in Appendix E. The results show that the ratios of the measured pulse energy to the energy limit under different situations are very small and well within the exposure limit. The highest of them all is 2.17% which occurs at 541 nm under single pulse exposure (Rule 1). Therefore the system can potentially be used on the eye in practical situation.

6.7 Summary

A HS-PAS system to acquire the normalised OAC spectrum of the highly-absorbing samples is proposed in this chapter. This allows the characterisation of healthy iris and uveal melanoma in the iris using PA method, which can be used to detect diseases. Such characterisation is important to determine the optimal wavelength for PA excitation such that there is good contrast difference between healthy iris and uveal melanoma. The use of an OAC reference removes the need to perform spectral calibrations for the optical components between the photodiode and the sample, which can have wavelength-dependent transmittance and reflectance. Optical components can be removed or added to the PA setup

without the need to perform yet another spectral calibration. Normalised OAC spectra made up of 461 spectral bands were acquired from 410 nm - 870 nm with a spectral interval of 1 nm. The proposed methodology enables precise wavelength selection of 1-nm resolution, which can be used for spectroscopic or multispectral imaging applications. During the process of building a library of the normalised OAC spectrum, it is desired to use wavelengths which are close of the order of 1 nm, so that any detailed spectral signature would be detected [104]. The selection of wavelengths is very important in multispectral PA imaging where few wavelengths are used in detecting and differentiating the different components in a sample. A good selection of these wavelengths help to improve the temporal resolution while acquiring PA signals that can be used to give reliable results [104]. This can only happen when detailed spectral information is available so that the precise wavelengths can be selected.

It is also illustrated that this proposed HS-PAS system and methodology can be employed to determine the normalised OAC spectrum of highly-absorbing targets by using fluorescent microsphere suspensions and iris region of the eye as test samples. The acquired spectra from a healthy iris can be used as reference spectra for ocular disease diagnosis. It is expected that this proposed approach can also be adopted for the measurement of other highly-absorbing materials for a variety of applications.

The next chapter entails a hybrid-modality imaging system based on a commercial clinical ultrasound imaging system using a linear-array UST and a tunable pulsed laser for optical excitation. The integrated system uses photoacoustic and ultrasound imaging for ocular imaging to provide complementary absorption and structural information of the eye.

Chapter 7: Hybrid-modality ocular imaging

using clinical ultrasound system and nanosecond pulsed laser

Hybrid-modality imaging is a special type of multimodality imaging which has been significantly used in the recent past to harness the strengths of different imaging methods as well as to furnish complementary information beyond that provided by any individual method. A hybrid-modality imaging system based on a commercial clinical ultrasound imaging system using a linear-array ultrasound transducer and a tunable nanosecond pulsed laser for optical excitation is presented. The integrated system uses photoacoustic and ultrasound imaging for ocular imaging to provide complementary absorption and structural information of the eye. In this system, B-mode images from photoacoustic and ultrasound imaging are acquired at 10 Hz and about 40 Hz, respectively. A linear-array ultrasound transducer makes the system much faster compared to other ocular imaging systems using a single-element ultrasound transducer to form B-mode images. The results show that the proposed instrumentation is able to incorporate photoacoustic and ultrasound imaging in a single setting. The feasibility and efficiency of this developed probe system is illustrated using enucleated porcine eye samples. It is demonstrated that photoacoustic imaging could capture photoacoustic signals from the iris, anterior lens surface, and posterior pole, while ultrasound imaging could accomplish the mapping of the eye to reveal the structures like the cornea, anterior chamber, lens, iris, and posterior pole. Gold nanocages are then used as photoacoustic contrast agents. Photoacoustic images are taken from porcine eye samples before and after the introduction of gold nanocage solution above the iris. The photoacoustic signal from the iris is stronger after introducing gold nanocages.

7.1 Introduction

Photoacoustic imaging (PAI) is commonly integrated with ultrasound imaging (USI) because both imaging modalities are detecting acoustic waves using an ultrasound transducer (UST). USI has already been widely used and accepted in many clinical applications. By combining these two imaging modalities, it also makes it easier for clinicians to accept PAI as an emerging imaging modality [22]. It has been reported in

literature that systems use single-element USTs, which require mechanical scanning to form a B-mode image [14,144]. Such scanning makes the overall speed of the system slow and more susceptible to motion artefacts, thus reducing the image quality. From these perspectives, this chapter details a novel integrated hybrid-modality imaging platform, where a fast clinical USI system is easily integrated with a tunable nanosecond pulsed laser. The developed system uses a linear-array UST, which facilitates the data acquired in each scan to form a B-mode image devoid of mechanical scanning. In this integrated hybrid-modality imaging system, the optical absorption-based information is available through PAI and structural information through USI. The system's ability to derive such complementary information is demonstrated by using enucleated porcine eye sample as test sample. Hybrid-modality imaging of the eye can provide complementary and clinically useful information, so that a better diagnostic evaluation and confirmation of uveal melanoma can be made by clinicians. The system can find clinical applications in the diagnosis of uveal melanoma, a type of ocular cancer which can arise in the iris leading to blindness or death [15]. PAI can be used to differentiate between the healthy iris and tumour, and to determine the tumour size, spread, and type. By combining this information with that obtained from USI, the location of the tumour with respect to other ocular structures is revealed. Gold nanocages are then used as photoacoustic (PA) contrast agents, which represent bioconjugated gold nanocages with specific binding to detect uveal melanoma in the iris.

7.2 Instrumentation of hybrid-modality ocular imaging system

The experimental setup shown in Fig. 7.1 consists of a commercial clinical USI scanner (UltraVision 64B Research Platform, WinProbe) and a laptop with dedicated software (UltraVision Control Panel) to process the data acquired from the UST and to display the

PA and ultrasound (US) images. The UltraVision software comes with several functions that are commonly seen in many clinical USI systems, such as the selection of image depth and focal depth, time-gain compensation, and spatial compounding. PA and US images are acquired using a 128-element linear-array UST set within a clinical-style imaging probe (L15, WinProbe). The UST has a centre frequency of 15 MHz and a bandwidth of more than 60%. The elements have a pitch of 0.1 mm, thus the probe has an azimuthal length (width of view at the surface) of 12.8 mm for USI. The azimuthal length for PAI is only 6.4 mm as only the centre 64 elements in the UST are used for PAI. The WinProbe USI system can be seen in Appendix F. A tunable nanosecond pulsed laser (Vibrant 355II, Opotek Inc) is used as the optical excitation for PAI. It operates at 10 Hz and the wavelength selection and the output intensity of the pulsed laser can be controlled using the laptop. Whenever a pulse is fired by the laser, Q-switch synchronization of the laser sends a trigger to the scanner.

Fig. 7.1: Instrumentation of hybrid-modality imaging system.

US excitations/pulses are delivered by the linear-array UST and the detected echoes form US images. The scanner calculates the number of US images that it can produce between

the PA triggers, which is affected by user-defined parameters such as the imaging depth and settings for spatial compounding. It creates these US images and waits for the next PA trigger. Once the scanner receives the PA trigger from the laser, US excitation from the UST stops and the UST will only receive signals for a short time (about 50 to 200 μ s). The time duration is dependent on the imaging depth and during this time the signals acquired are used to form a PA image. USI resumes after the PA image is formed. In this study, PAI and USI run at 10 Hz and about 40 Hz, respectively. An exposure time of 8 s is sufficient to acquire the data for each set of measurement.

For demonstration purposes, the porcine eye sample was held in place by a holder and aligned to face upwards. The lens of the eye is placed about 2 cm away from the UST and the focusing depth of the US mode is also set to 2 cm. The line illumination is placed across the diameter of the eye and on its anterior segment. The UST is placed above this line and is just in contact with the water surface. The fluence of the line illumination on the eye is not uniform. On a flat surface, the fluence of the line illumination is higher near the centre of the line. When applied to the eye, the fluence is further affected by the shape of the eye.

7.3 Preparation of porcine eye samples

Due to the similarities in morphology between porcine and human eyes [149], the porcine eye sample is chosen as an *ex vivo* animal model in this study. Porcine eye samples have been used in vision sciences' research involving corneal transplant and glaucoma [149,150]. The main differences between the porcine and human eyes include the absence of the Bowman's layer in the porcine eye and that the cornea thickness of the porcine eye is twice that of humans [151].

Six randomly selected enucleated porcine eyes (*Sus scrofa domestica*) were acquired from local abattoir. Extraocular tissues such as the conjunctiva and lacrimal gland were removed from the samples. The samples were placed and transported on ice until the experiments began to maintain their “freshness.” Only samples which were found to be without any sign of deterioration during visual inspection were used for testing. The eye samples were tested within 6 hours of death. A total of eight porcine eye samples have been used in this study, all of which were conducted according to Nanyang Technological University’s regulations on biosafety and the regulations of Agri-Food & Veterinary Authority of Singapore.

7.4 Results and discussion

The spatial resolutions of the hybrid-modality imaging system in both the PA and US modes are shown. Imaging of enucleated porcine eye samples were carried out and the adherence to guideline on exposure limit to laser radiation of the iris was verified. Thereafter, the results show the images of porcine eye samples before and after the introduction of gold nanocages.

7.4.1 Spatial resolution

A human hair was measured to have a diameter of about 105 μm and used as a test target to evaluate the system’s axial and lateral resolution in both the PA and US modes. The target was placed horizontally and perpendicular to the UST. Similar to the imaging setup for the *ex vivo* porcine eye sample, the hair was kept at a distance of about 2 cm away from the UST, with the focusing depth of the US mode set to 2 cm. The PA and US images of the human hair are shown in Fig. 7.2.

Fig. 7.2: (a) PA and (b) US images of human hair.

The maximum amplitudes of the signals in both images were located, and Gaussian fitting was applied to the amplitude profiles along the vertical and horizontal directions. The full widths at half maximum of the Gaussian fittings were applied to quantify the system's axial and lateral resolutions (Fig. 7.3). The PA mode was found to have axial and lateral resolutions of about 0.25 mm and 1.35 mm, respectively. While the US mode was found to have axial and lateral resolutions of about 0.42 mm and 0.97 mm, respectively. Axial resolutions in both modes were better than their lateral resolutions where signals appear to spread more in the horizontal directions (Fig. 7.2). The resolutions in the two modes were different and one reason may be because the US mode had a function to determine the focusing depth, but not for the PA mode.

Fig. 7.3: Normalised Gaussian fittings of axial and lateral profiles of (a) PA and (b) US images of human hair.

7.4.2 Imaging of porcine eye samples

7.4.2.1 Long illumination

Using the UltraVision software, the system was able to capture and display PA and US images side by side on the laptop. The settings could also be configured such that a pseudo-coloured PA image is overlaid on the US image. In order to improve the contrast of PA images, they were processed in MATLAB® after the experiment before being presented in the following sections. However, no change was made to the US images.

Fig. 7.4(a) shows the schematic of the eye and Fig. 7.4(b) shows the US image of the enucleated porcine eye sample. By comparing Fig. 7.4(b) to Fig. 7.4(a), the ocular features in the US image include the cornea, anterior chamber, lens, iris, and posterior pole.

Fig. 7.4: (a) Schematic diagram of eye and (b) US image of porcine eye sample.

The images in Fig. 7.5 were acquired from the same eye sample and at the same position as in Fig. 7.4. Fig. 7.5(a) shows the PA image using a 500-nm pulsed laser illumination. It can be observed that the PA signals were produced from certain specific regions, as shown in the image. Without a clear understanding of the structures of the eye, it can be difficult to determine the exact location from which the PA signals were produced. In Fig. 7.5 (b), the PA image is overlaid onto the US image to form a combined image. Both the ocular structural features from USI and the absorption-based information from PAI appear in one image. With the combined image, it is now evident that strong PA signals were produced from the pigmented iris, and weaker PA signals from the anterior lens surface (ALS) and the posterior pole. As the fluence of the line illumination was not uniform, it is not suitable to compare the properties of the iris, ALS, and posterior pole.

Fig. 7.5: (a) PA and (b) combined PA/US images of enucleated porcine eye sample.

7.4.2.2 Short illumination for constant fluence

This study was conducted so that the lens and iris were separately illuminated under the same fluence. The line illumination was reduced to a shorter length of about 3 mm by blocking the path of the illumination from the two ends. Only the centre region of the line illumination was allowed to pass. The UST was positioned such that its centre axis was in line with this illumination. First, the eye sample was moved into position such that the centre of the lens was illuminated. The short illumination was much smaller than the lens, thus only the lens was illuminated and not the iris. The results are shown in Fig. 7.6(a-b). Next, the eye sample was repositioned such that the iris appeared in the middle of the US image. Only the iris region was illuminated and the results are shown in Fig. 7.6(c-d).

Fig. 7.6: (a) PA and (b) combined images with lens illumination, and (c) PA and (d) combined images with iris illumination.

The results in Fig. 7.6, where the lens and iris were illuminated separately, were acquired under the same temperature, wavelength and fluence. In this case, Eq. (2.4) is reduced to

$$P_0 \propto \Gamma\mu, \quad (7.1)$$

where P_0 is the strength of the PA wave, Γ is the dimensionless Grüneisen parameter and μ is the optical absorption coefficient.

From Fig. 7.6(a-b), it can be observed that the top PA signals originated from the ALS, and the bottom PA signal came from the posterior pole. From Fig. 7.6(c-d), a PA signal was acquired from the pigmented iris. The imaging depths of the ALS and iris are similar, and thus their fluences are estimated to be about the same. By comparing the amplitude of the PA signals when fluence was the same [Eq. (7.1)], it can be deduced that $(\Gamma\mu)_{\text{Iris}}$ is much higher than $(\Gamma\mu)_{\text{ALS}}$. This is attributed to the pigmented iris containing melanin, which is highly optically absorbing compared to the optically clear lens ($\mu_{\text{Iris}} \gg \mu_{\text{ALS}}$) [14].

As the short illumination travelled across the lens and further into the eye toward the posterior pole, the illumination path could not be estimated reliably. Therefore, the fluence

on the posterior pole is not known. Though the ALS and posterior pole produced PA signals of comparable amplitude, no additional information can be drawn from the obtained results. It is known that the posterior pole contains blood vessels that are highly absorbing, which could be producing PA waves [144].

PA signals from the pigmented iris containing melanin and from the posterior pole of the eye which contains blood vessels are expected, as both melanin and blood are highly absorbing. However, it is unexpected for the ALS with low optical absorption to be producing PA signals if only the absorption properties of the lens are considered. The result of the ALS producing PA waves is similar to those already reported [14]. Although this phenomenon is still not clarified, possible explanations include the post-mortem changes and an unidentified chromophore [14], all of which are related to μ . However, it can also be seen from Eq. (7.1) that the amplitude of the PA wave is not only directly proportional to μ , but also to Γ . Therefore, both μ - and Γ -related aspects have to be investigated to determine how the ALS generates PA waves. The effect of Γ on the amplitude of the PA wave should not be neglected.

In the present study, a linear-array UST was used for the detection of the PA and reflected US waves. The vertical distance between the linear-array UST and the eye increases when moving away from the centre of the eye. For both PAI and USI, this space needs to be filled up by an acoustic coupling medium, such as the ultrasound gel which is commonly used in clinical environments. If this space can be made smaller, the amount of gel needed can be reduced and it will become more clinically convenient. This issue can be overcome if a curved-array UST is used. An optical fiber can also be used to deliver the

optical excitation for PAI when the curved-array UST is placed closer to the eye. Future modifications to the developed system will be along these directions.

7.4.2.3 Reproducible experimental results

Fig. 7.7 shows the combined PA/US images from four sets of experimental results acquired from different porcine eye samples. Fig. 7.7(a) is also Fig. 7.6(d) that appeared earlier in Sec. 7.4.2.2. It can be observed from Fig. 7.7 that the combined images are similar to each other. The experimental PA and US results are therefore reproducible.

Fig. 7.7: (a), (b), (c) and (d) are four sets of combined images from porcine eye samples.

7.4.2.4 Adherence to guideline on exposure limit to laser radiation

For potential diagnostic clinical applications to detect uveal melanoma in the iris, the exposure limit (EL) of the system is subjected to guidelines defined by International Commission on Non-Ionizing Radiation Protection for the skin [148]. For this purpose, where the illumination is targeted at the iris and not the cornea and retina, the EL for skin is used as the guideline to protect the anterior parts of the eye [148]. Table 7.1 shows the parameters used for the below calculations for repetitive pulse exposures for skin.

Table 7.1: Parameters for calculations of repetitive pulse exposures^a.

λ (nm)	PRF (Hz)	t_{Pulse} (ns)	T_{Max} (s)	C_A	Area (m ²)
500	10	5	8	1.0 for 400 nm $\leq \lambda < 700$ nm	9.62113E-06 ^b

^a λ : Excitation wavelength; PRF: Pulse repetition frequency; t_{Pulse} : Pulse duration; T_{Max} : Total exposure duration; C_A : Spectral correction factor related to melanin absorption.

^bArea of 3.5-mm diameter limiting aperture.

Two general rules are applied when using repetition pulsed systems and the EL for skin exposure. Rule 1 states that the exposure from a single pulse should not exceed the EL for one pulse of that pulse duration. In this case, the pulse EL is

$$EL_{\text{SP}} = 200C_A = 200 \text{ J/m}^2. \quad (7.2)$$

Considering a 3.5-mm diameter limiting aperture, the pulse energy EL is

$$EL_1 = EL_{\text{SP}} \times \text{Area} = 1.92 \text{ mJ}. \quad (7.3)$$

Rule 2 states that the exposure from any group of pulses delivered in time T_{Max} should not exceed the EL for time T_{Max} . For a T_{Max} of 8 s, the EL is

$$EL_{\text{Rep}} = 11C_A T_{\text{Max}}^{0.25} = 18.50 \text{ kJ/m}^2. \quad (7.4)$$

Considering a 3.5-mm diameter limiting aperture and that there are multiple pulses in T_{Max} , the pulse energy EL is

$$EL_2 = \frac{EL_{\text{Rep}} \times \text{Area}}{T_{\text{Max}} \times \text{PRF}} = 2.22 \text{ mJ}. \quad (7.5)$$

The pulse energy EL for a single pulse is lower than that of an exposure for the full 8 s, thus EL_1 is used in this study. Using the same experimental settings, the pulse energy was measured using a power meter (Nova Display and 12AV1, Ophir) and found to be 0.131 mJ, which is only about 7% of EL_1 . Therefore, the current configuration can potentially be used for diagnostic applications to detect uveal melanoma in the iris.

7.4.3 Imaging of porcine eye samples with gold nanocages as contrast agent

As mentioned in Sec. 2.3.5.2i, gold nanoparticles have many attractive properties such that they have garnered a lot of attention and have been used in PAI [117]. Common gold

nanoparticles include nanorods, nanospheres and nanocubes. Gold nanoparticles are optically tunable over a broad spectrum from the near-ultraviolet to mid-infrared [120,121], by ways such as altering its shape (aspect ratio) [122] and the relative dimensions [120,121]. When tuned to the near-infrared region where tissue transmissivity is high, imaging of thick tissues is allowed due to the deep penetration of light.

Gold nanocages (AuNcgs) represent a novel class of nanostructures with hollow interiors and porous walls [152], possessing some properties that are superior when compared to gold nanorods and nanohexapods. Of these three gold nanostructures, AuNcgs have the highest photothermal conversion efficiency per gold atom. AuNcgs and gold nanohexapods have the same photothermal stability which are much higher than that of gold nanorods [153].

Here, AuNcgs were produced using microwave oven heating technology, with a synthesis time of about few seconds compared to conventionally produced AuNcgs with synthesis time of several hours. The synthesis and characterisation of the quick-synthesised AuNcgs can be found in Appendix G. Mechanical precision in the control of temperature and power output of microwave heating are two major advantages of this method apart from the remarkable decrease in the time of reaction compared to established synthesis methods [154,155]. The initial PA experiments using AuNcgs can be found in Appendix H.

Gold NP has a surface coating of gold which is biocompatible, making it a suitable CA for bio-imaging [118,121]. Although AuNcgs have been used contrast agents in the PAI of cerebral cortex and skin melanomas [156,157], this is the first time AuNcgs are used as PA contrast agents for ocular imaging. This is demonstrated by showing its PA contrast enhancement in enucleated porcine eye samples. It is reported that bioconjugated AuNcgs have specific binding to the surfaces of cancer cells [158]. The injected AuNcgs in this

experiment represent tagged AuNcgs to uveal melanoma in the iris, causing an increase in localised optical absorption. Such changes can be detected using PAI by comparing the images obtained prior and after the introduction of AuNcgs. The enhanced PA signals due to the presence of the AuNcgs can be used as an indication of the location and size of uveal melanoma.

The experimental setup in this section is very similar to that used in Sec. 7.2, except that another 128-element linear-array UST set within a clinical-style imaging probe (L8, WinProbe) was used. The UST has a frequency of 5 MHz - 10 MHz. The elements have a pitch of 0.3 mm, thus the probe has an azimuthal length of 38.4 mm for USI. For PAI, the azimuthal length is only 19.2 mm as only the centre 64 elements in the UST are used.

Four porcine eye samples were used in this study. They were prepared as mentioned in Sec. 7.3. The eyes were positioned towards the right of the UST so that the left iris appeared at the centre of the images. Two sets of imaging were conducted by taking 50 PA and 50 US images of the porcine eye samples. In this study, the laser beam with wavelength of 500 nm and size of 3 mm was used for excitation at the iris.

The first imaging produced a set of PA and US images representing a healthy eye sample (before the introduction of AuNcgs into the eye). It was then removed from the water tank and the AuNcg solution was slowly injected into the region above the left iris of the eye sample (Appendix I). This was used to simulate uveal melanoma tagged by bioconjugated AuNcgs in the iris region.

The acquired images were processed offline using a custom-written MATLAB® script. Each set of 50 images were averaged to form a representative image with reduced random noises. Signals of weak intensity in the representative PA images were also removed for

better clarity. For each eye sample, the two representative PA images were normalised with respect to the strongest signal in the PA image before the AuNcg solution was introduced. The combined PA/US images of all porcine eye samples can be seen in Fig. 7.8-Fig. 7.11.

Fig. 7.8: Combined images of porcine eye sample A (a) before and (b) after injection of AuNcg solution.

Fig. 7.9: Combined images of porcine eye sample B (a) before and (b) after injection of AuNcg solution.

Fig. 7.10: Combined images of porcine eye sample C (a) before and (b) after injection of AuNcg solution.

Fig. 7.11: Combined images of porcine eye sample D (a) before and (b) after injection of AuNcg solution.

It can be observed from Fig. 7.8-Fig. 7.11 that PA signals were generated from the iris on the left using the structural features revealed by the US images. The signals that appear from outside the eye region [Fig. 7.8(b) and Fig. 7.11(a)] are presumably attributed to system-generated random noises.

An area of about $0.35 \times 0.35 \text{ mm}^2$ was selected as the interrogation region and each set of 50 PA images was considered for the analysis. These areas were selected from the illuminated iris region where PA signals were produced. The amplitudes of the PA signals within each area were average to represent the strength of the PA signals as presented by the system. It was found that after the injection of AuNcg solution into the region above the left

iris of the eye samples, the strength of the PA signals in the images for samples A to D increased by $46.3 \pm 14.3\%$, $81.4 \pm 16.7\%$, $57.9 \pm 14.8\%$ and $17.6 \pm 17.2\%$, respectively (Fig. 7.12). These results show that the AuNcgs can potentially be used as a PA contrast agent in ocular imaging. Specific targeting of AuNcgs to markers of diseases can potentially be used to identify diseases [158] such as uveal melanoma by using PAI for diagnostic applications.

Fig. 7.12: Increase in strength of PA signals after injection of AuNcg solution.

7.5 Summary

In this chapter, a hybrid-modality imaging system based on a commercial clinical USI platform with a linear-array UST set within a clinical-style imaging probe and a tunable nanosecond pulsed laser, is presented. The integrated system uses PAI and USI to provide complementary absorption and structural information, respectively. Photoacoustic and ultrasound B-mode image are acquired at the rate of 10 Hz and about 40 Hz (based on the user-defined parameters), respectively. Using a linear-array ultrasound transducer, each B-mode images captured by this system requires only one scan, compared to other scanning ocular imaging systems using a single element ultrasound transducer. The system and the proposed methodology are validated by using enucleated porcine eyes as the test samples.

The results showed that the proposed instrumentation is able to perform PAI and USI under the same setting. PAI could successfully capture PA signals from the iris, ALS, and posterior pole, whereas USI could accomplish the mapping of the eye to reveal structures like the cornea, anterior chamber, lens, iris, and posterior pole. Hybrid-modality imaging of the eye can provide complementary and clinically useful information, so that a better diagnostic evaluation and confirmation of uveal melanoma can be made by clinicians. This system and the proposed methodology are expected to be used as a preclinical imaging system in ocular imaging and other relevant diagnostic medical applications.

AuNcgs were used as PA contrast agents, which represented bioconjugated AuNcgs with specific binding to detect uveal melanoma in the iris. PAI was conducted on enucleated porcine eye samples before and after the introduction of AuNcg solution above the iris. It was clearly evident from the obtained data and images that the strength of the PA signals from the iris increased after AuNcgs were introduced, which can potentially be used as an indication of the location and size of uveal melanoma.

In order to increase the system performance, one of the future work directions of research should be in improving the spatial resolution. This can be achieved by using better image processing algorithms or adding a galvanometer to scan the focused beam for PAI [159].

Chapter 8: Conclusions and recommendations for future work

This chapter begins with the discussion on the conclusions of this research thesis. This is followed by the major contributions and ends with the recommendations for future work directions.

8.1 Conclusions

A pushbroom hyperspectral imager, which incorporates a video camera not only for direct video imaging but also for user-selectable region of interest within the field of view of the video camera, has been proposed and successfully demonstrated. Custom-developed software allows scanning to take place only within the selected region of interest. The additional benefits of using the video camera for user-selectable region of interest include no unwanted scanning, data acquisition time as well as the data size are minimised. A smaller data size in turn translates to a shorter computational time in data processing and analysis. The minimum and maximum fields of view of the video camera are about $4.32 \times 5.76 \text{ mm}^2$ (working distance of about 21.5 cm) and $5.17 \times 6.89 \text{ mm}^2$ (working distance of about 23.8 cm), respectively. The system has a maximum spectral range covering the visible to near-infrared wavelength band from 400 nm - 1000 nm, and can detect 756 spectral bands within this spectral range. The maximum achievable lateral resolution of this system at maximum zoom without using any image enhancement is about 40 μm . The experiments conducted with the bio- and fluorescent phantom samples also demonstrate that the developed pushbroom hyperspectral imager can be used for both reflection and fluorescence based imaging modalities. This is the main hyperspectral imaging platform for

probe-based imaging in the colon to detect cancer progression of different stages by integrating it with a flexible probe scheme.

A pushbroom hyperspectral imaging probe-based on spatial-scanning method has been conceptualised and developed for the first time. The imaging probe is an assembly of a gradient index lens and an imaging fiber optic bundle. The system offers 756 spectral bands for detection within the full spectrum range of the system. Lateral resolution of the system is wavelength-dependent and this can be seen in both the theoretical simulation using Zemax and the follow up experimental validation. The lateral resolution along the horizontal and vertical directions at 505 nm is about 40 μm . In order to demonstrate the diagnostic bio-imaging capability as a proof of concept, a chicken breast tissue with blood clot was used as test sample. Distinct reflectance spectra of the chicken breast tissue and blood clot were acquired for analysis. The pushbroom hyperspectral imaging probe can be used on samples that are difficult to reach and close to being stationary. The scope of existing table-top pushbroom hyperspectral imager is extended by enabling it to perform endoscopic bio-imaging using a flexible imaging probe. The pushbroom hyperspectral imaging probe can be used to image the colon for the detection of cancer progression of different stages. Hundreds of spectral images can also be acquired for disease diagnosis applications to give an efficient data library which is not possible by other conventional endoscopic means. It is envisaged that this probe expected to be very useful as an *in vivo* optical biopsy probe in the near future.

A snapshot hyperspectral video-endoscope is conceptualised and developed using a custom-fabricated two-dimensional to one-dimensional fiber bundle. It converts a pushbroom hyperspectral imager into a snapshot configuration. The fiber bundle is flexible

and has a small distal end enabling it to be used as an imaging probe that can be inserted into the colon for minimally invasive and *in vivo* investigations for the detection of cancer. A snapshot hyperspectral video-endoscope is developed. A frame rate of about 6.16 Hz can be attained, and each frame was converted into a three-dimensional datacube with 756 spectral bands. The three-dimensional datacubes and intensity mappings provide vast amount of information, which includes the spatial features (shape and size), spectral signatures, speed and direction of the imaged samples. The lateral resolutions of the system along the horizontal and vertical directions were found to be 157.49 μm and 99.21 μm , respectively. Bio- and fluorescent phantom tissue samples representing different stages of cancer growth were imaged in reflectance and fluorescence imaging modalities for proof of concept studies.

A hyperspectral photoacoustic spectroscopy system, to acquire the normalised optical absorption coefficient spectrum of highly-absorbing samples, is proposed and developed. This allows the characterisation of healthy iris and uveal melanoma in the iris using the photoacoustic method, which can be used to detect diseases. Such characterisation is important to determine the optimal wavelength for photoacoustic excitation such that there is good contrast difference between healthy iris and uveal melanoma. The use of an optical absorption coefficient reference removes the need to perform spectral calibrations for the optical components between the photodiode and the sample, which can have wavelength-dependent transmittance and reflectance. Optical components can be removed or added to the photoacoustic setup without the need to perform yet another spectral calibration. Both theoretical and experimental investigations were carried out. Normalised optical absorption coefficient spectra from 410 nm - 870 nm were acquired with a spectral resolution of 1 nm,

which can be used for spectroscopic or multispectral imaging applications. The proposed system and methodology were employed to determine the normalised optical absorption coefficient spectrum of the highly-absorbing samples such as fluorescent microsphere suspensions and iris region of the eye.

A probe-based hybrid-modality imaging system was configured and illustrated with test samples to demonstrate the feasibility of the system for ocular imaging applications. This system is based on a commercial clinical ultrasound imaging platform with a linear-array ultrasound transducer set within a clinical-style imaging probe and a tunable nanosecond pulsed laser. The integrated system uses photoacoustic imaging and ultrasound imaging to provide complementary absorption and structural information, respectively. Photoacoustic and ultrasound B-mode images are acquired at the rate of 10 Hz and about 40 Hz (based on the user-defined parameters), respectively. Using a linear-array ultrasound transducer, each B-mode images captured by this system requires only one scan, compared to other scanning ocular imaging systems using a single element ultrasound transducer. The system and the proposed methodology are validated by using enucleated porcine eyes as the test samples. The results showed that the proposed instrumentation is able to perform photoacoustic imaging and ultrasound imaging under the same setting. Photoacoustic imaging could successfully capture photoacoustic signals from the iris, anterior lens surface, and posterior pole, whereas ultrasound imaging could accomplish the mapping of the eye to reveal structures like the cornea, anterior chamber, lens, iris, and posterior pole. Hybrid-modality imaging of the eye can provide complementary and clinically useful information, so that a better diagnostic evaluation and confirmation of uveal melanoma can be made by clinicians. Gold nanocages were used as photoacoustic contrast agents, which represented gold

nanocages with specifically binding to uveal melanoma in the iris. Photoacoustic images were taken from enucleated porcine eye samples before and after the introduction of gold nanocage solution above the iris. The photoacoustic signals from the iris increased after gold nanocages were introduced, which can potentially be used as an indication of the location and size of uveal melanoma. This system and the proposed methodology are expected to be used as a preclinical ocular imaging system and other relevant diagnostic medical applications.

8.2 Major contributions

The major contributions of the thesis are as follow:

- (i) A novel pushbroom hyperspectral imager which incorporates a video camera for user-selectable region of interest is conceptualised, developed and demonstrated. The methods and formulas used for calibration and electronic hardware interfacing have been discussed. This concept prevents unwanted scanning and minimises data acquisition time, data size and computational time. The system has a maximum field of view of about $4.32 \times 5.76 \text{ mm}^2$ and lateral resolution of about $40 \text{ }\mu\text{m}$. It can detect 756 spectral bands within the spectral range of 400 nm - 1000 nm.
- (ii) A spatial-scanning hyperspectral imaging probe is proposed and demonstrated for the first time, using the pushbroom method by a motorised stage. This is achieved by integrating an imaging probe with a table-top pushbroom hyperspectral imager. The scope of existing table-top pushbroom hyperspectral imager is extended, and can now perform probe-based or endoscopic imaging. The probe-based system has a circular field of view of about 1 mm diameter and lateral resolution of about $40 \text{ }\mu\text{m}$. It can detect 756 spectral bands within the spectral range of 400 nm - 1000 nm. Using

Zemax, theoretical modelling and simulation were conducted on the gradient index lens and the results show that chromatic aberration causes large variations in the quality of spectral images at different wavelengths.

- (iii) A snapshot hyperspectral video-endoscope has been conceptualised and verified experimentally. It captures 756 spectral bands within the spectral range of 400 nm - 1000 nm, significantly more wavelength bands than existing hyperspectral endoscopes. It is the first to use a two-dimensional to one-dimensional fiber bundle to realise the snapshot endoscopic configuration. The system has a field of view of about $1.11 \times 1.32 \text{ mm}^2$ and lateral resolutions along the horizontal and vertical directions are about 157.49 μm and 99.21 μm , respectively. Datacubes are acquired at a rate of 6.16 Hz.
- (iv) The concept of using an optical absorption coefficient reference in photoacoustic spectroscopy is proposed and demonstrated. This concept is based on the theoretical manipulation of the equation governing the generation of photoacoustic pressure, by comparing the measurements between the sample and optical absorption coefficient reference. The system also performs photoacoustic measurements from 410 nm - 870 nm with spectral interval of 1 nm, capturing data from 461 wavelength bands (hyperspectral) so that the detailed spectral signatures can be acquired.
- (v) A hybrid-modality snapshot imager is designed and developed for ocular imaging, using a commercial clinical ultrasound imaging system integrated with a pulsed nanosecond laser. Complementary absorption-based and structural information of the eye are acquired using photoacoustic (10 Hz) and ultrasound imaging (about 40 Hz), respectively. Using the L15 ultrasound transducer (WinProbe), the ultrasound mode

has axial and lateral resolutions of about 0.42 mm and 0.97 mm, respectively. While the photoacoustic mode has axial and lateral resolutions of about 0.25 mm and 1.35 mm, respectively.

- (vi) Gold nanocages were used as photoacoustic contrast agents for the first time in ocular imaging. The obtained data and images show that the strength of the photoacoustic signals from the iris increased after gold nanocages are introduced. Specific targeting of the gold nanocages to markers of diseases [158] can possibly be used to detect diseases by using photoacoustic imaging as the imaging modality.

8.3 Recommendations for future work

The work done in this thesis has potential for numerous studies to be carried out in the future. The following are the recommendations for some identified future work directions.

- The table-top pushbroom hyperspectral imager has a stationary line of view where hyperspectral measurements are conducted. However, wide-field illumination is applied on the sample. A focused line-illumination on the sample which coincides with the line of view of the hyperspectral measurements can be integrated into this system. This can potentially increase the contrast and spatial resolution of the system, as the line-illumination ensures that only light from the line of view is detected. Otherwise, light from the sample close to the line of view may also be captured by the system when wide-field illumination is used. Another benefit is a reduction in exposure time due to higher fluence, resulting in a shorter data acquisition time.
- Initial work has been done on the use of hyperspectral imaging to authenticate polymer banknotes (Appendix J), using the table-top pushbroom hyperspectral imager with a maximum field of view of about $5.17 \times 6.89 \text{ mm}^2$. This can be sufficient in cases

where small-area hyperspectral imaging is required on a few regions of the banknotes. However, in cases where hyperspectral imaging of the entire back or front side of the banknote is required, the current configuration is not suitable. Therefore, the pushbroom hyperspectral imager can be reconfigured so that it becomes better suited for such an application. The size of a local banknote in general circulation can go up to $9 \times 18 \text{ cm}^2$ (\$10,000 banknote of Portrait Series, Singapore). Thus the line of view during hyperspectral measurement has to be at least 9 cm and the stage translation has to be at least 18 cm, so that the entire back or front side of the banknote can be imaged in one measurement. A more time-efficient way is for the line of view to be at least 18 cm so that the stage translation only needs to be 9 cm. These can be achieved by adjusting the optics of the system and integrating a new motorised stage with the required translation to the system.

- For the hyperspectral imaging systems using spatial-scanning probe and snapshot video-endoscope, digital processing techniques to compensate for the effect of curvature for samples with large curvature region as the interrogation area can be investigated. Also, configurations that allow for the illumination to be integrated into the same fiber bundle can be looked into. A beam splitter can be used to reflect the illumination into the proximal end of the fiber bundle. Illumination will exit from the distal end of the fiber bundle and fall onto the sample. The reflection or fluorescence from the sample is then collected by the same fiber bundle from its distal end, exit its proximal end and pass through the beam splitter towards the detector camera (Fig. 8.1). Alternatively, separate optical fibers can be used only for the delivery of light to the distal end of the fiber bundle for sample illumination. These allow the probes to

deliver illumination to the sample directly to become more practical and suitable for clinical environments.

Fig. 8.1: Beam splitter for delivery of illumination.

- The snapshot hyperspectral video-endoscope can be enhanced by using fiberlets of smaller diameter, so that more fiberlets can be packed along the one-dimensional end of the fiber bundle. This allows spectral information from more spatial points to be collected and data acquisition by the sensor array becomes more efficient. The spatial resolution of the hyperspectral image is also improved at the same time. Another improvement to the system is to add a miniaturised lens to the distal end of the fiber bundle to acquire focused images from the sample for better spatial resolution. These enable the probe to image diseases of smaller dimensions. These improvements when integrated in a new probe will enable it to become a potentially usable device. In a new probe design where the outer and core diameters of the fiberlet are reduced by half, the number of pixels can double from 100 to 200. If a miniaturised lens with a $0.5\times$ magnification is now added, the resolution will remain of the order of $100\ \mu\text{m}$ but with about four times the image area. When the image area is still insufficient, another method that can be used is to use the output of multiple spectrometers. The use of four spectrometers in a system has been reportedly [56]. When these strategies are used, the fiber bundle can have 784 fiberlets arranged in a 28×28 hexagonal array

with an image area of about 10.86 mm^2 , which is about 7.4 times that of the current. Furthermore, another set of fibers for illumination can be added to the probe, while existing fiberlets continue as collection fibers. This completes the probe as it also delivers light to illuminate the sample directly. One design of such an improved probe can be seen in Fig. 8.2 and Fig. 8.3. The frame rate of the system can also be increased from the current approximate 6.16 Hz to 20 Hz so that it becomes real-time, which can be done by using a detector camera with a faster readout rate and having a lower exposure time. A faster frame rate is preferred so that more information is captured for better diagnostic evaluation and confirmation by clinicians. It also helps to negate the blurring effects in the images due to motion artifact.

Fig. 8.2: Improved two-dimensional to one-dimensional fiber bundle probe showing front-views of all ends.

Fig. 8.3: Side-view of distal end of improved fiber bundle probe.

- A tunable pulsed laser with a pulse repetition frequency of at least 20 Hz (currently 10 Hz) can be used for both the hyperspectral photoacoustic spectroscopy and hybrid-

modality snapshot imager for ocular imaging. This helps by reducing the data acquisition time of the hyperspectral photoacoustic spectroscopy system by half. In the hybrid-modality snapshot imager, the photoacoustic images can be captured in real-time. This captures more photoacoustic information and reduces the blurring effects in the images due to motion artifact for better diagnostic evaluation and confirmation by clinicians.

- The hybrid-modality snapshot imager currently uses optical components to direct the optical excitation from the pulsed laser to the sample. Light guide can be integrated with the ultrasound transducer probe so that the optical excitation can be delivered directly from the probe to the sample, making the system more practical and convenient for clinicians. The spatial resolution of the system can also be enhanced by using better image processing algorithms.
- Gold nanoparticles and nanoparticles with gold coating are reported as bio-compatible, making it a suitable contrast agent for bio-imaging [118,121]. Gold nanocages also fall in this group where the outer layer of the cage is made of gold [152]. Further studies are planned with clinicians as future work direction.

It is envisaged that the major findings and original contributions of this thesis are expected to contribute well towards diagnostic bio-imaging applications pertaining to colon cancer and uveal melanoma in the near future.

Appendices

Appendix A: MATLAB® script to arrange two-dimensional data to three-dimensional datacube

HyperSpec saves the information of each datacube in multiple files of the .txt format. The two-dimensional data acquired in each scan are placed sequentially one after the other in the two dimensional .txt files. The saved files are imported and processed by an in-house written script in MATLAB® to arrange the two-dimensional data to a single three-dimensional datacube, as shown below.

```
clear all;
close all;

numfiles = 7;

% Open each .txt file as MATLAB® data file
for a = 1:numfiles
    myfilename = sprintf('%d.txt', a);
    eval(sprintf('Data_%d = dlmread(myfilename);', a));
    eval(sprintf('file = size(Data_%d);', a));
    eval(sprintf('filerow%d = file(1,1);', a));

    if a == 1
        eval(sprintf('filerowtot = filerow%d;', a));
    else
        eval(sprintf('filerowtot = filerowtot + filerow%d;', a));
    end
end

file = size(Data_1);
filerow = file(1,1);
wl_row = filerow/histc(Data_1(:,1),-1)-1;
N = histc(Data_1(:,1),-1);

% Save wavelength of each band
Wavelength_table = Data_1(2:wl_row+1,1);
filecol = file(1,2);

% Create stitched two-dimensional file
Data_2D = zeros(filerowtot, filecol);
```

```
% Stitching files
for b = 1:numfiles
    startrow = 1 + filerow1*(b-1);
    if b ~= numfiles
        endrow = filerow1*b;
    else
        endrow = filerowtot;
    end
    Data_2D(startrow:endrow,1:filecol) = eval(sprintf('Data_%d', b));
end

% Determine size of three-dimensional data
c = size(Data_2D);
x = c(1,2)-1;
y = c(1,1)/(wl_row+1);

% Delete wavelength column
Data_2D(:,1) = [];

% Delete x-pixel number rows
for stack = 0:y-1
    Data_2D(wl_row*stack+1,:) = [];
end

% To form 3D data
Data_3D = zeros(y, x, wl_row);

for z1 = 1:wl_row
    for y1 = 1:y
        Data_3D(y1,1:x,z1) = Data_2D(wl_row*(y1-1)+z1,1:x);
    end
end
```

Appendix B: MATLAB® script to plot cut-datacube

Many types of plots can be made available from one datacube. These include spectrum plot, images at different wavelength bands and datacube. The MATLAB® script to plot a cut-datacube such as the one in Fig. 3.14(a), is shown below. The figure from such a script shows a portion of the datacube that is removed so that the internal features can be seen. The colour image is also placed on the cut-datacube so that the information in the datacube can be related to the colour image.

```
m = 1:band:wl_row;
m = floor(m);

Z_tick_label = round(Wavelength_table(m,1));

figure('color', [1 1 1])
n = slice(Data_3D, [1 ceil(x/2)], [ceil(y/2) 1], [1 ceil(wl_row/2)]);
hold on
n1 = slice(Data_3D(1:y, 1:x, 1:ceil(wl_row/2)), x, y, []);
n2 = slice(Data_3D(1:y, 1:ceil(x/2), 1:wl_row), [], y, []);
n3 = slice(Data_3D(1:ceil(y/2), 1:x, 1:wl_row), x, [], []);
set(n, 'EdgeColor','none', 'FaceColor','interp')
set(n1, 'EdgeColor','none', 'FaceColor','interp')
set(n2, 'EdgeColor','none', 'FaceColor','interp')
set(n3, 'EdgeColor','none', 'FaceColor','interp')
title('Cut data-cube ', 'FontSize', 25, 'FontWeight', 'bold')
xlabel(['Andor EMCCD x-pixel: ', sprintf('%0.2f', x_step), '\mum'], 'FontSize', 20)
ylabel(['Stage step: ', sprintf('%0.2f', stage_step), '\mum'], 'FontSize', 20)
zlabel('Wavelength (nm)', 'FontSize', 20)
set(gca, 'YDir', 'reverse', 'XTick', X_tick_label, 'XTickLabel', X_tick_label, 'YTick',
Y_tick_label, 'YTickLabel', Y_tick_label, 'ZTick', m, 'ZTickLabel', Z_tick_label,
'DataAspectRatio', AR, 'FontSize', 15)
colormap jet
ylabel(colorbar, 'Intensity count', 'FontSize', 20)
xImage = [1 x; 1 x]; % x-coor for image corners
yImage = [1 1; ceil(y/2) ceil(y/2)]; % y-coor for image corners
zImage = [wl_row+1 wl_row+1; wl_row+1 wl_row+1]; % z-coor for image corners
surf(xImage, yImage, zImage, 'CData', imgtop, 'FaceColor', 'texturemap', 'linestyle', 'none')
xImage = [1 ceil(x/2); 1 ceil(x/2)]; % x-coor for image corners
yImage = [ceil(y/2) ceil(y/2); y y]; % y-coor for image corners
zImage = [wl_row+1 wl_row+1; wl_row+1 wl_row+1]; % z-coor for image corners
```

```

surf(xImage, yImage, zImage, 'CData', imgbot, 'FaceColor', 'texturemap', 'linestyle', 'none')
plot3([x, x], [ceil(y/2), y], [ceil(wl_row/2), ceil(wl_row/2)], 'k', 'Linewidth', 2)
plot3([ceil(x/2), ceil(x/2)], [ceil(y/2), y], [ceil(wl_row/2), ceil(wl_row/2)], 'k', 'Linewidth',
2)
plot3([ceil(x/2), ceil(x/2)], [ceil(y/2), y], [wl_row, wl_row], 'k', 'Linewidth', 2)
plot3([ceil(x/2), x], [y, y], [ceil(wl_row/2), ceil(wl_row/2)], 'k', 'Linewidth', 2)
plot3([ceil(x/2), x], [ceil(y/2), ceil(y/2)], [ceil(wl_row/2), ceil(wl_row/2)], 'k', 'Linewidth',
2)
plot3([ceil(x/2), x], [ceil(y/2), ceil(y/2)], [wl_row, wl_row], 'k', 'Linewidth', 2)
plot3([ceil(x/2), ceil(x/2)], [y, y], [ceil(wl_row/2), wl_row], 'k', 'Linewidth', 2)
plot3([ceil(x/2), ceil(x/2)], [ceil(y/2), ceil(y/2)], [ceil(wl_row/2), wl_row], 'k', 'Linewidth',
2)
plot3([x, x], [ceil(y/2), ceil(y/2)], [ceil(wl_row/2), wl_row], 'k', 'Linewidth', 2)
plot3([x, x], [y, y], [1, ceil(wl_row/2)], 'k', 'Linewidth', 2)
plot3([ceil(x/2), 1], [y, y], [wl_row, wl_row], 'k', 'Linewidth', 2)
plot3([x, x], [1, ceil(y/2)], [wl_row, wl_row], 'k', 'Linewidth', 2)
plot3([x, x], [1, y], [1, 1], 'k', 'Linewidth', 2)
plot3([1, x], [y, y], [1, 1], 'k', 'Linewidth', 2)
plot3([1.1, 1.1], [y, y], [1, wl_row], 'k', 'Linewidth', 2)
plot3([x, x], [1.1, 1.1], [1, wl_row], 'k', 'Linewidth', 2)
axis('on', 'tight')
view(45,30)

```

Appendix C: Spot diagrams using gradient index lens at optimised object-lens distance

Each wavelength behaves differently as it moves through the gradient index lens (Chapter 4). This causes wavelength-dependent optical characteristics on the distal end-face of the fiber bundle. At the optimized object-lens distance (about 0.316 mm), the effect of the different representative wavelengths on the image quality is investigated using Zemax. The on-axis root-mean-square radius with centroid reference is used as a measure of image quality. The spot diagrams below show the on-axis root-mean-square radii with centroid references for 400 nm, 700 nm and 850 nm, which are 41.440 μm , 14.872 μm and 24.018 μm , respectively.

Fig. C.1: Zemax spot diagram of 400 nm on distal end-face of fiber bundle.

Fig. C.2: Zemax spot diagram of 700 nm on distal end-face of fiber bundle.

Fig. C.3: Zemax spot diagram of 850 nm on distal end-face of fiber bundle.

Appendix D: LabVIEW® software for photoacoustic experiments

A LabVIEW® software (control panel in Fig. D.1) was developed to control and synchronise the laser, 3-axis motorised stage and digitizer for the photoacoustic experiments in Chapter 6. It also saved the averaged signals from the transducer and photodiode.

Fig. D.1: Control panel of developed LabVIEW® software.

Appendix E: Adherence to guideline on exposure limit to laser radiation

For potential diagnostic clinical applications to characterise healthy and diseased sites in the iris for the detection of uveal melanoma (Sec. 6.6.3), the exposure limit (EL) of the system is subjected to guidelines defined by International Commission on Non-Ionizing Radiation Protection [148]. The detailed calculations and relevant analysis regarding this for different wavelengths are given below.

Table E.1 shows the parameters used for the below calculations for repetitive pulse exposures for skin.

Table E.1: Parameters for calculations of repetitive pulse exposures^a.

PRF (Hz)	t_{Pulse} (ns)	T_{Train} (s)	T_{Max} (s)	Area (m ²)
10	5	4	2530	1.132E-06

^aPRF: Pulse repetition frequency; t_{Pulse} : Pulse duration; T_{Train} : Exposure duration for each wavelength; T_{Max} : Total exposure duration.

The spectral correction factor related to melanin absorption (C_A) is

$$C_A = \begin{cases} 1.0, & 400 \text{ nm} \leq \lambda < 700 \text{ nm} \\ 10^{0.002(\lambda/1 \text{ nm} - 700)}, & 700 \text{ nm} \leq \lambda < 1050 \text{ nm} \end{cases} \quad (\text{E.1})$$

where λ is the optical excitation wavelength.

Two general rules are applied when using repetition pulsed systems and the EL for skin exposure. Rule 1 states that the exposure from a single pulse should not exceed the EL for one pulse of that pulse duration. In this case, the pulse EL is

$$\text{EL}_{\text{SP}} = 200C_A \text{ J/m}^2. \quad (\text{E.2})$$

Considering the laser spot size, the pulse energy EL is

$$\text{EL}_1 = \text{EL}_{\text{SP}} \times \text{Area}. \quad (\text{E.3})$$

The EL_1 calculated for the selected wavelengths are in Table E.2. Ratio_1 is the ratio of the measured pulse energy (Table 6.1) to EL_1 of each wavelength.

Table E.2: EL_1 and $Ratio_1$.

λ (nm)	C_A	EL_1 (μJ)	$Ratio_1$ (%)
410	1.000	226.5	1.00
475	1.000	226.5	1.35
541	1.000	226.5	2.17
620	1.000	226.5	1.92
700	1.000	226.5	1.20
740	1.202	272.3	1.26
800	1.585	358.9	1.58
870	2.188	495.5	1.01

Rule 2 states that the exposure from any group of pulses, or sub-group of pulses in a train, should not exceed the EL for the time duration. For a T_{Train} of 4 s for each wavelength, the EL is

$$EL_{Rep,A} = 11C_A T_{Train}^{0.25} \text{ kJ/m}^2. \quad (E.4)$$

Considering the laser spot size and that there are multiple pulses in T_{Train} , the pulse energy EL is

$$EL_{2,A} = \frac{EL_{Rep,A} \times Area}{T_{Train} \times PRF}. \quad (E.5)$$

The $EL_{2,A}$ calculated for the selected wavelengths are in Table E.3. $Ratio_{2,A}$ is the ratio of the measured pulse energy (Table 6.1) to $EL_{2,A}$ of each wavelength.

Table E.3: $EL_{2,A}$ and $Ratio_{2,A}$.

λ (nm)	$EL_{2,A}$ (μJ)	$Ratio_{2,A}$ (%)
410	440.4	0.51
475	440.4	0.69
541	440.4	1.11
620	440.4	0.99
700	440.4	0.62
740	529.4	0.65
800	697.9	0.81
870	963.4	0.52

Within the T_{Max} of 2530 s, 461 wavelengths have actually been used. Nevertheless, for the T_{Max} of 2530 s in a situation where only one wavelength is used, the EL is calculated:

$$EL_{Rep,B} = 2.0C_A \text{ kW/m}^2. \quad (E.6)$$

Considering the laser spot size and that there are multiple pulses in T_{Max} , the pulse energy EL is

$$EL_{2,B} = \frac{EL_{Rep,B} \times Area \times T_{Max}}{\lambda_{Total} \times T_{Train} \times PRF}. \quad (E.7)$$

where λ_{Total} is the total number of wavelengths. The $EL_{2,B}$ calculated for the selected wavelengths are in Table E.4, which shows the pulse energy EL when only one wavelength replaces all the other wavelengths. However, 461 wavelengths have actually been used. Therefore, the highest measured pulse energy of 5.669 μJ at 800 nm (Table 6.1) is used to compare with the lowest $EL_{2,B}$ ($Ratio_{2,B}$) in this safety analysis. $Ratio_{2,B}$ is found to be about 1.8%.

Table E.4: $EL_{2,B}$.

λ (nm)	$EL_{2,B}$ (μJ)
410	310.7
475	310.7
541	310.7
620	310.7
700	310.7
740	373.6
800	492.4
870	679.8

The ratios of the measured pulse energy (Table 6.1) to the energy limit under different situations are very small and well within the exposure limit. The highest of them all is 2.17% which occurs at 541 nm under single pulse exposure (Rule 1). Therefore the system can potentially be used on the eye in practical situation.

Appendix F: WinProbe ultrasound imaging system

Chapter 7 used an integrated hybrid-modality imaging platform, where a fast clinical ultrasound imaging system is easily integrated with a tunable nanosecond pulsed laser. The ultrasound imaging system is an UltraVision 64B Research Platform commercial clinical scanner from WinProbe (Fig. F.1) and came with a laptop with dedicated UltraVision software (control panel in Fig. F.2).

Fig. F.1: Photograph of WinProbe scanner shown with ultrasound transducers used.

Fig. F.2: Control panel of UltraVision software.

Two 128-element linear-array ultrasound transducers had been used. They are set within a clinical-style imaging probe. The first transducer is L15 from WinProbe [Fig. F.3(a)]. It has a centre frequency of 15 MHz and a bandwidth of more than 60%. The elements have a pitch of 0.1 mm, thus the probe has an azimuthal length of 12.8 mm for ultrasound imaging. The next transducer is L8 also from WinProbe [Fig. F.3(b)]. This transducer has a frequency of 5 MHz - 10 MHz. The elements have a pitch of 0.3 mm, thus the probe has an azimuthal length of 38.4 mm for ultrasound imaging.

Fig. F.3: (a) L15 and (b) L8 clinical ultrasound transducers from WinProbe.

Appendix G: Synthesis and characterisation of gold nanocages

Chemicals and instruments

Polyvinylpyrrolidone (PVP) of average $M_w \sim 55,000$ (Cat.# 856568), Sodium sulfide nonahydrate (Cat.# 208043) and Gold (III) chloride trihydrate (Cat.# G4022-1G) were procured from Sigma-Aldrich, Japan. Ethylene glycol (Cat.# 14114-00) and silver nitrate (Cat.# 37075-30) were purchased from Kanto chemicals, Japan. All other chemicals and reagents used were of analytical grade.

Transmission electron microscopy (TEM) images were recorded with TEM of JEOL. Scanning electron microscopy (SEM) images were taken using SU6600 of Hitachi. Ultraviolet-visible absorption spectra of as-synthesised particles were measured with spectrophotometer (DU730, Beckman Coulter).

Synthesis of gold nanocages (AuNcgs)

AuNcgs were produced using microwave oven heating technology, with synthesis time of few seconds compared to conventionally produced AuNcgs with synthesis time of several hours. Mechanical precision in the control of temperature and power output of microwave heating are two major advantages of this method apart from the remarkable decrease in the time of reaction compared to established synthesis method [154,155].

Silver nanocubes (AgNcbs) were synthesised based on the microwave assisted polyol method mentioned elsewhere [160]. Briefly, 10 ml of ethylene glycol solution of $250 \mu\text{M}$ Na_2S was vigorously stirred after adding 0.075 M PVP. The mixture was injected drop wise using a syringe into 10 ml of ethylene glycol solution of AgNO_3 (0.05 M) under constant magnetic stirring. During which, the solution turned wine-coloured due to the formation of Ag_2S . Wine-coloured solution was intermittently heated (stop & start method) in a

microwave and swirled manually for thorough mixing between the heating steps. Heating experiment was conducted in a microwave oven (YJ-50H8, LG Electric) operating at frequency of 50 Hz, power consumption of 1000 W and rated high frequency output of 500 W. Khaki-coloured solution containing AgNcbs was formed within seconds of reaction. Nanocubes were washed several times before galvanic replacement with HAuCl_4 solution took place. Galvanic replacement reaction was conducted using 5 ml of 0.1 mM HAuCl_4 solution with 550 μl of as-synthesised AgNcbs in the microwave oven. AgNcb solution was first mixed with 5 ml of 9 mM aqueous solution of PVP before introducing gold solution. The mixture was heated in the microwave oven intermittently until a stable pale-purple colour was obtained. AuNcgs were washed several times with water and 1:1 ethanol/water mixture and dispersed in deionised water.

Characterisation of AuNcgs

Fig. G.1(a) shows the TEM image of an AuNcg with holes in the faces and corners with the corresponding fast Fourier transform (FFT) image shown in the inset. TEM characterisation revealed the hollow nature of the AuNcg showing a thick-walled cubical box pattern. The wall thickness of AuNcgs was measured to be 5 ± 2 nm with an average edge length of about 65 nm. Atomic resolution image of one of the corners of AuNcg was recorded and presented in Fig. G.1(b). Line profile of the FFT data generated shows the inter-plane distance of the gold crystal [Fig. G.1(c)]. Line profile [Fig. G.1(d)] of the well-defined lattice fringes from the selected area of Fig. G.1(b) shows the d-spacing of $2\text{\AA}$, which can be indexed to (200) planes of the face-centred cubic lattice structure of gold [161].

Fig. G.1: (a) TEM image of AuNcg with inset showing the FFT image, (b) zoom-in of one corner of AuNcg, (c) line profile of FFT image in (a), and (d) line profile of TEM image of AuNcg shown in (b).

Depending upon the degree of passivation by PVP over AuNcgs, the dispersity of the AuNcgs differs. However on thorough washing and ultrasonication, well mono-dispersed AuNcgs were collected.

SEM image shows well-dispersed, corner truncated AuNcgs with pores in the faces and corners [Fig. G.2(a)]. Inverted greyscale SEM image shows the transparent white zones which correspond to the holes present in AuNcgs [Fig. G.2(b)]. Ultraviolet-visible spectra of AgNcbs and AuNcgs show that the evolution of hollow AuNcgs from solid AgNcbs shifts the peak absorption towards the near-infrared region (Fig. G.3).

Fig. G.2: (a) SEM and (a) inverted greyscale SEM images of AuNcgs.

Fig. G.3: Ultraviolet-visible absorbance spectra of AgNcbs and AuNcgs.

Appendix H: Initial photoacoustic experiments using gold nanocages

Verification of photoacoustic (PA) waves generation

The gold nanocages (AuNcgs) were suspended in a solution. In order to prove that the AuNcgs in the solution were able to produce PA signals, an experiment measuring the strength of PA signals produced by AuNcg solutions of varying concentrations was conducted. The optical absorption coefficient (μ) of a solution is dependent on its molar absorption ε and concentration (Conc), as seen in Eqn. (H.1) [85,110].

$$\mu = \varepsilon \cdot \text{Conc.} \quad (\text{H.1})$$

Substituting Eqn. (H.1) into Eqn. (2.4) [31,93,104] gives Eqn. (H.2).

$$P_0(\text{Temp}, \lambda) = \Gamma(\text{Temp})F(\lambda)\mu(\lambda), \quad (2.4)$$

$$P_0(\text{Temp}, \lambda) = \Gamma(\text{Temp})F(\lambda)\varepsilon(\lambda)\text{Conc.} \quad (\text{H.2})$$

where P_0 is the initial pressure rise of the PA wave, Γ is the dimensionless Grüneisen parameter, F is the optical fluence, Temp is the temperature in medium and λ is the optical excitation wavelength.

Considering that only an excitation wavelength is used, Temp , Γ and ε being constants and F corrected, Eqn. (H.2) becomes Eqn. (H.3), showing that if the AuNcg can produce PA signals, the strength of the PA signals is directly proportional to its concentration.

$$P_0 \propto \text{Conc.} \quad (\text{H.3})$$

The experimental setup to capture PA signals generated by various concentrations of the quick-synthesised AuNcgs is the same as shown in Fig. 6.1(b), except that in this case the ultrasound transducer (UST) was in contact with the cuvette (104-10-40, Hellma Analytics). Excitation wavelength of 800 nm was used. The signals were averaged over 100 pulses for each measurement to improve the signal-to-noise ratio. Five measurements were acquired for each concentration of AuNcg solution.

The cuvette was initially filled with 1.5 ml of the AuNcg solution using a single-channel pipettor (4075, LambdaTM Plus Corning). The initial concentration of the AuNcg solution was 100% and with this concentration, measurements were carried out for five times. This is continued for a total of nine concentrations: 100%, 80%, 64%, 51.2%, 40.96%, 32.77%, 26.21%, 20.97% and 16.78%. A control experiment was also carried out with 0% AuNcg concentration using only deionized water. A total of 50 measurements (five for each of ten concentrations) were acquired.

Data acquired from the experiments were processed offline using in-house written script in MATLAB®. Data processing for the experiment started with Hilbert transformation of the UST signals and compensation for fluence variations using signals from the photodiode. The processed signals at this stage from measurement 1 of four selected AuNcg concentrations are shown in Fig. H.1. Hilbert transformation is widely used in analytical signal analysis to pick up the envelopes of vibration signals [107]. These signals were corrected for any background PA signals using the mean value of the signal from the sample with 0% AuNcg concentration. The mean and standard deviation of the maximum values in the measurements of the concentrations (P_{Max}) were calculated and normalized.

Fig. H.1: Processed signals of four selected AuNcgs concentrations.

The means and standard deviations of P_{Max} at varying AuNcg concentrations are plotted against the concentration, as shown in Fig. H.2. The line of best fit with zero-intercept is also plotted. The slope of the line of best fit and R^2 value were also determined to be 1.0235 and 0.9938, respectively. The R^2 value of 0.9938 is very close to 1, indicating that the experimental data are very close to the line of best fit. The gradient of the line of best fit of 1.0235 is very close to 1, indicating that the amplitude of the PA waves detected by the UST is directly proportional to the AuNcg concentration. This is consistent with Eqn. (H.3) and proves that the signals acquired from the experiment were due to the PA waves generated by the AuNcgs in the solution.

Fig. H.2: P_{Max} against AuNcg concentration.

Photoacoustic imaging (PAI) of varying concentrations of AuNcg solution in tubings

Three transparent flexible plastic tubings (S3 E-3603, Tygon) with inner and outer diameters of 3.2 mm and 4.8 mm, respectively were used in this experiment. They were held in place from the ends and placed at about 7.5 mm apart using an acrylic holder (Fig. H.3). The left tubing was filled with deionised water, the centre tubing was filled with a mixture of deionised water and AuNcg solution (1:1 volumetric ratio) and the right tubing was filled with AuNcg solution. They were then left to settle for about 30 minutes.

Fig. H.3: (a) Three tubings held in place by acrylic holder and (b) close-up of tubings.

The experimental setup in this section is very similar to that used in Sec. 7.4.3. During the experiment, the tubings were partially submerged in the water and the linear-array UST was placed transverse to and above the lowermost point of the three tubings. PA excitation was delivered to one tubing at a time to ensure that constant excitation fluence was delivered to each tubing. Also, the linear-array UST was only able to capture PA images across 19.2 mm, while the width of the arrangement of the tubings were longer at about 20 mm. In order to get PA signals in this experiment, the excitation beam with wavelength of 500 nm and size of 5 mm was used. First, the left tubing which was placed below the UST was excited and PA images were taken. This is followed by exciting the center and right tubings to obtain the relevant PA images. 50 PA and 50 US images were captured when each tubing was imaged.

Similar to Sec. 7.4.3, 50 US and 50 PA images were captured for each set of measurement to form representative images of the tubings. The combined PA/US images can be seen in Fig. H.4.

Fig. H.4: Combined PA/US images of excited (a) left, (b) centre and (c) right tubings.

It can be seen from Fig. H.4(a) that a weak PA signal was acquired from the top of the left tubing. In Fig. H.4(b), a weak PA signal was also acquired from the top of the centre tubing, but two other PA signals were also observed from the top and bottom of its internal section. The same was observed in Fig. H.4(c), but PA signals from the internal section of the right tubing are observed to be larger in amplitude and more intense. The PA signals from the internal section of the centre and right tubings are attributed to the presence of AuNcgs in the solution in the tubings.

Two areas from the centre tubing where PA signals are observed (top and bottom of tubing's internal section) are selected. From the interrogation area of $\sim 0.35 \times 0.35 \text{ mm}^2$, the values are selected and averaged to represent the strength of the PA signals due to the AuNcgs. The same was done for the right tubing. The representative strength of the PA signals from the centre tubing to that of the right tubing has a ratio of 0.827:1, which is not proportional to the AuNcg concentration in the tubings of 0.5:1. This could be due to how the PA images are presented by the system, where the algorithms used may not be linear

(approximate log compression). However, the trend of a higher AuNcg concentration giving stronger PA signals still holds. It was also observed after the experiment that some AuNcgs adhered to the internal surface of the tubing while AuNcg aggregates were seen in the solution.

Appendix I: Preparation of porcine eye sample for injection of gold nanocage solution

After the first measurement, the porcine eye sample was removed from the water tank and about 0.15 ml of gold nanocage solution was slowly injected into it, just above the iris on the left (Fig. I.1). The porcine eye sample was then left untouched for about 20 minutes so that the gold nanocage solution can settle.

Fig. I.1: Injection of gold nanocage solution above left iris of porcine eye sample.

Appendix J: Hyperspectral imaging to authenticate polymer banknotes

Introduction

The use of polymer banknotes is becoming more popular these days and is even replacing paper-based banknotes, as they offer many additional security features such as transparent windows. They are also more durable and remain more consistent compared to paper notes. Security features such as watermarks and fluorescent features may be present in genuine notes to help in its identification. However, with better printing and reproduction equipment made available, counterfeiters are able to reduce the differences between genuine and counterfeit notes. It therefore becomes more challenging to identify counterfeit notes. Hyperspectral imaging (HSI) can be used to authenticate polymer notes but this is not widely reported.

In this context, this study demonstrates the use of HSI on polymer notes for authentication purposes. The pushbroom hyperspectral (HS) imager as mentioned in Chapter 3 is adopted in this study. The instrumentation is the same as in Sec. 3.2, except that an additional near-infrared lamp (HP3616, Philips) was used as a light source.

The flexibility in selecting region of interest (ROI) allows large-area ROI to be selected for imaging and small-area ROI to be chosen when only the spectra are required. A library of reference spectra acquired from different parts of the genuine polymer notes can be created. These reference spectra serve as the authentication platform that can help identifying the counterfeit polymer notes by analysing the differences between them.

In order to get the Reflectance data, the Sample data were corrected by dark reference (Dark) and white reference (White) using

$$\text{Reflectance}(x, y, \lambda) = \text{Smooth} \left[\frac{\text{Sample}(x, y, \lambda) - \text{Dark}(x, y, \lambda)}{\text{White}(x, y, \lambda) - \text{Dark}(x, y, \lambda)} \right] \times 0.99. \quad (\text{J.1})$$

Sample data were acquired when the note was imaged. Dark data were acquired when the light sources were turned off and the forelens was covered. It represents the image with dark current noise where the reflectance was 0%. White data were acquired by imaging the 99% reflectance standard (SRS-99-010, Labsphere). x and y refer to the spatial dimensions in the horizontal and vertical directions, respectively. λ is the wavelength and Smooth is the 11-point moving average in the spectral direction for spectrum smoothing. Data processing was done offline using a custom-written MATLAB® script.

Acquiring reference spectra from genuine polymer banknotes

Three randomly chosen circulated genuine Singapore polymer \$10 banknotes were used as reference banknotes (RefNote1, RefNote2 and RefNote3), and four randomly chosen regions (Lion: gold patch on front design, Dot: top right corner of front design, Number: bottom of back design and Cap: central region of back design) were imaged using the HS imager. Each region on each reference banknote was measured twice. Thus six spectra were used to build the reference spectrum for each region (three banknotes and two measurements). The imaged ROIs of RefNote1 had varying sizes (about 8.4 mm² - 14.5 mm²) and are shown in Fig. J.1.

After data processing, each set of measurement gives a reflectance datacube. Fig. J.2 shows the cut-datacube of Dot and Number for measurement 1 of RefNote1 to reveal more features within the reflectance datacube. Each horizontal slice of the datacube is the reflectance mapping of the ROI at a wavelength. Thus 756 reflectance mappings can be acquired from each datacube. A reflectance spectrum is obtained by acquiring the data from each spatial point down the datacube.

Fig. J.1: Locations and ROIs of (a) Lion, (b) Dot, (c) Number and (d) Cap of RefNote1.

Fig. J.2: Cut-datacubes of (a) Dot and (b) Number of measurement 1 of RefNote1.

A reflectance spectrum for each measurement is acquired by averaging the spectra of 5×5 selected spatial points of the datacube, which is about $54 \times 52 \mu\text{m}^2$ on the ROI. The white square in each ROI of Fig. J.1 indicates the spot where the reflectance spectrum is acquired from each reference banknote. The reference spectrum (defined as Reference as shown in Fig. J.3) of each region is the average of the six spectra collected from the three reference banknotes with two measurements each, and are shown in Fig. J.3.

Fig. J.3: #Reflectance spectra from reference banknotes of (a) Lion, (b) Dot, (c) Number and (d) Cap.

#The first and second numbers of the legend represent the reference banknote and measurement, respectively.

The differences between the two spectra acquired from the same note and region (repeated measurements) are very low. This causes each sub-figure in Fig. J.3 to look like they have less than seven spectra, when there are seven spectra each. The results acquired using the HS imager are highly reproducible. The variations in the spectra acquired from the same region but different reference banknotes are presumably due to the inherent differences in the reference banknotes examined in this study. These may be due to differences in circulation period and frequency and the conditions in which the notes were handled and kept.

The average standard deviations of the reference spectra of Lion, Dot, Number and Cap are $\pm 2.075\%$, $\pm 1.045\%$, $\pm 0.472\%$ and $\pm 1.180\%$, respectively. The reference spectrum of Lion has the largest standard deviations. Its ROI appears to be powder-coated, unlike others which use ink or dye. Therefore the consistency of the ROI of Lion is not as high. The standard deviations of the reference spectrum from a large set of reference banknotes can be a good indicator of whether the ROI is consistent and suitable to be used for authentication.

Most of the other imaged ROIs in this study were of a much smaller area compared to those seen in Fig. J.1. Some were as small as about $54 \times 52 \mu\text{m}^2$ (0.0028 mm^2), and just sufficiently large to acquire a datacube with only 5×5 spatial points to get the reflectance spectra. This is made possible with the use of the developed flexible HS imager incorporating a video camera for a user-selectable ROI, minimizing measurement time, data size and computational time.

Authentication of polymer banknotes

To demonstrate the ability of using HSI to authenticate polymer banknotes, the reference spectra that were earlier acquired are compared against the spectra acquired from other genuine and simulated counterfeit test samples. The three reference banknotes were scanned at a resolution of 1200 dots per inch. Only the surrounding area of each region was laser-printed (xerography) in colour (Color LaserJet CM6040, HP) and used as simulated counterfeit test samples (CF1, CF2 and CF3). Another three circulated genuine notes (G1, G2 and G3) acted as genuine test samples. A measurement was done on each sample and region. Similar to how the spectra were acquired from the reference banknotes, a reflectance spectrum was acquired by averaging 5×5 spatial points from the same position where the reference spectra were acquired. The ROIs of CF1 had varying sizes (about 6.2 mm^2 - 12.7 mm^2) and are shown in Fig. J.4. The white square within each ROI represents the location from which the reflectance spectrum is acquired.

Fig. J.4: ROIs of (a) Lion, (b) Dot, (c) Number and (d) Cap of CF1.

The spectra acquired from the test samples for authentication are shown together with the reference spectra in Fig. J.5 and Fig. J.6. In this study, the root-mean-square error (RMSE) analysis was used to determine the amount of differences between each spectrum and its respective reference. It gives a single value which is easy to understand and sufficient to perform authentication in this study. An RMSE of a low value indicates that the amount of differences between the sample's spectrum and the reference is low. This implies that the same ROI in both the sample and the reference have very similar spectral characteristics. If authentication is done based on this ROI only, the sample is classified as a genuine note. In the opposite case, the sample is classified as a counterfeit note. The RMSE between the spectra from all test samples and their respective references are summarized in Table J.1.

Fig. J.5: ^ Reflectance spectra from genuine banknotes and reference spectra of a) Lion, b) Dot, c) Number and d) Cap.

^G1, G2 and G3 refer to the three test genuine banknotes.

Fig. J.6: *Reflectance spectra from simulated counterfeit banknotes and reference spectra of a) Lion, b) Dot, c) Number and d) Cap.

*CF1, CF2 and CF3 refer to the three simulated counterfeit banknotes.

Table J.1: Summary of reflectance RMSE (%).

Region	Genuine test samples				Simulated counterfeit test samples			
	G1	G2	G3	Average	CF1	CF2	CF3	Average
Lion	0.99	4.31	2.58	2.63	11.58	11.45	15.38	12.80
Dot	2.72	2.34	2.25	2.44	12.72	13.18	11.19	12.36
Number	1.39	3.80	2.28	2.49	6.51	6.18	7.55	6.75
Cap	1.36	4.26	5.13	3.58	18.50	22.45	18.79	19.91

The results in Table J.1 show that the reflectance spectra from the genuine test samples have some differences when compared to the reference spectra. This is expected as the genuine notes are not exactly the same, which may be due to factors such as the differences in circulation period and frequency and the conditions in which the notes were handled and kept. The table also shows that the RMSEs from the genuine notes are significantly lower than that from the counterfeit notes in different ways. Firstly, even the lowest RMSE from the counterfeit test samples is significantly higher than the highest RMSE from the genuine

test samples in each region. The lowest RMSE from the counterfeit test sample is about 166%, 311%, 63% and 261% more than the highest RMSE from the genuine note for Lion, Dot, Number and Cap, respectively. Secondly, the Average columns in Table J.1 show that for each region, the RMSEs of the spectra from the genuine test banknotes with respect to their reference spectra are much lower compared to those obtained from the simulated counterfeit test banknotes.

For each region, user can define an RMSE for authentication ($RMSE_{Aut}$). Any RMSE lower or higher than $RMSE_{Aut}$ is considered as a genuine or counterfeit banknote, respectively. Each region has its own $RMSE_{Aut}$ as the consistency in each of them varies. By setting an $RMSE_{Aut}$ of about 4.5%, 3%, 4% and 5.5% for Lion, Dot, Number and Cap respectively, each region in the genuine test samples is classified as being from a genuine note, while each region in the simulated counterfeit test samples is classified as being from a counterfeit note. The results show that the proposed methodology of using HSI for data acquisition to build a library of reference spectra, coupled with using RMSE for data analysis, can be used to authenticate polymer banknotes effectively against the simulated counterfeit notes used in this study.

List of publications

Journal papers (Published)

- [1] **H.-T. Lim** and V.M. Murukeshan, “Pushbroom hyperspectral imaging system with selectable region of interest for medical imaging,” *Journal of Biomedical Optics* **20**(4), 046010 (2015).
- [2] **H.-T. Lim** and V.M. Murukeshan, “Hybrid-modality ocular imaging using a clinical ultrasound system and nanosecond pulsed laser,” *Journal of Medical Imaging* **2**(3), 036003 (2015).
- [3] **H.-T. Lim** and V.M. Murukeshan, “Spatial-scanning hyperspectral imaging probe for bio-imaging applications,” *Review of Scientific Instruments* **87**(3), 033707 (2016).
- [4] **H.-T. Lim** and V.M. Murukeshan, “A four-dimensional snapshot hyperspectral video-endoscope for bio-imaging applications,” *Scientific Reports* **6**, 24044 (2016).

Journal papers (under review)

- [1] **H.-T. Lim** and V.M. Murukeshan, “Hyperspectral photoacoustic spectroscopy of highly-absorbing samples for diagnostic ocular imaging applications,” Under review in International Journal of Optomechatronics.
- [2] S. Raveendran, **H.-T. Lim**, T. Maekawa, V.M. Murukeshan and D.S. Kumar, “Gold nanocages entering into the realm of high-contrast photoacoustic ocular imaging,” Under review in Nature Communications.
- [3] **H.-T. Lim** and V.M. Murukeshan, “Hyperspectral imaging of polymer banknotes for building and analysis of spectral library,” Under review in Optics and Lasers in Engineering.

- [4] **H.-T. Lim** and V.M. Murukeshan, "Design considerations and characterization of a flexible snapshot hyperspectral probe," Under review in Review of Scientific Instruments.

Conference papers (Published)

- [1] V.M. Murukeshan and **H.-T. Lim**, "Hybrid-modality high-resolution Imaging: for diagnostic biomedical imaging and sensing for disease diagnosis," in *Proc. SPIE* **9268**, 92680U (2014).
- [2] **H.-T. Lim** and V.M. Murukeshan, "Instrumentation challenges of a pushbroom hyperspectral imaging system for currency counterfeit applications," in *Proc. SPIE* **9524**, 95242I (2015).

References

- [1] R. Siegel, D. Naishadham and A. Jemal, "Cancer statistics, 2012," *CA: A Cancer Journal for Clinicians* **62**(1), 10-29 (2012).
- [2] J.J. Sung, J.Y. Lau, K.L. Goh and W.K. Leung, "Increasing incidence of colorectal cancer in Asia: Implications for screening," *The Lancet Oncology* **6**(11), 871-876 (2005).
- [3] T.D. Wang and J. Van Dam, "Optical biopsy: A new frontier in endoscopic detection and diagnosis," *Clinical Gastroenterology and Hepatology* **2**(9), 744-753 (2004).
- [4] A. Dhar, K.S. Johnson, M.R. Novelli, S.G. Bown, I.J. Bigio, L.B. Lovat *et al.*, "Elastic scattering spectroscopy for the diagnosis of colonic lesions: Initial results of a novel optical biopsy technique," *Gastrointestinal Endoscopy* **63**(2), 257-261 (2006).
- [5] S. Yoshida, S. Tanaka, M. Hirata, R. Mouri, I. Kaneko, S. Oka *et al.*, "Optical biopsy of GI lesions by reflectance-type laser-scanning confocal microscopy," *Gastrointestinal Endoscopy* **66**(1), 144-149 (2007).
- [6] K.D. Kochanek, J. Xu, S.L. Murphy, A.M. Miniño and H.-C. Kung, "Deaths: Final data for 2009," *National Vital Statistics Reports* **60**(3), 1 (2012).
- [7] J.V. Frangioni, "New technologies for human cancer imaging," *Journal of clinical oncology* **26**(24), 4012-4021 (2008).
- [8] G.N. Naumov, L.A. Akslen and J. Folkman, "Role of angiogenesis in human tumor dormancy: animal models of the angiogenic switch," *Cell Cycle* **5**(16), 1779-1787 (2006).
- [9] H.P. Lee, L. Chew, K.Y. Chow, E.Y. Loy, W. Ho and S. Low, "Trends in cancer incidence in Singapore, 2008-2012", National Registry of Diseases Office, Singapore (2013).
- [10] "Trends of colorectal cancer in Singapore, 2007-2011", National Registry of Diseases Office, Singapore (2013).
- [11] National Cancer Institute, "Colon Cancer Treatment (PDQ®): Stages of Colon Cancer", Updated: 21/03/2016, Accessed: 06/06/2016, (http://www.cancer.gov/types/colorectal/patient/colon-treatment-pdq#link/_112).
- [12] E. Ng, J.H. Tan, U.R. Acharya and J.S. Suri, Human Eye Imaging and Modeling, CRC Press (Boca Raton) 2012.

- [13] Schiller JS, Lucas JW and P. JA, "Summary health statistics for U.S. adults: National Health Interview Survey, 2011", National Center for Health Statistics, Vital and Health Statistics 10:256 (2012).
- [14] R.H. Silverman, F. Kong, Y. Chen, H.O. Lloyd, H.H. Kim, J.M. Cannata *et al.*, "High-resolution photoacoustic imaging of ocular tissues," *Ultrasound in Medicine & Biology* **36**(5), 733-742 (2010).
- [15] B. Damato, "Developments in the management of uveal melanoma," *Clinical & Experimental Ophthalmology* **32**(6), 639-647 (2004).
- [16] A. Nair, P. Thevenot, W. Hu and L. Tang, "Nanotechnology in the treatment and detection of intraocular cancers," *Journal of Biomedical Nanotechnology* **4**(4), 410-418 (2008).
- [17] C.L. Shields, S. Kaliki, S.U. Shah, W. Luo, M. Furuta and J.A. Shields, "Iris melanoma: Features and prognosis in 317 children and adults," *Journal of American Association for Pediatric Ophthalmology and Strabismus* **16**(1), 10-16 (2012).
- [18] H. Kashida and S.-e. Kudo, "Early colorectal cancer: Concept, diagnosis, and management," *International Journal of Clinical Oncology* **11**(1), 1-8 (2006).
- [19] D.K. Rex, "Maximizing detection of adenomas and cancers during colonoscopy," *The American Journal of Gastroenterology* **101**(12), 2866-2877 (2006).
- [20] N. Uedo, K. Higashino, R. Ishihara, Y. Takeuchi and H. Iishi, "Diagnosis of colonic adenomas by new autofluorescence imaging system: A pilot study," *Digestive Endoscopy* **19**(S1), S134-S138 (2007).
- [21] R.T. Kester, N. Bedard, L. Gao and T.S. Tkaczyk, "Real-time snapshot hyperspectral imaging endoscope," *Journal of Biomedical Optics* **16**(5), 056005 (2011).
- [22] L.V. Wang, "Prospects of photoacoustic tomography," *Medical Physics* **35**(12), 5758-5767 (2008).
- [23] J. Culver, W. Akers and S. Achilefu, "Multimodality molecular imaging with combined optical and SPECT/PET modalities," *Journal of Nuclear Medicine* **49**(2), 169-172 (2008).
- [24] M.A. Haidekker, *Medical Imaging Technology*, Springer (Berlin) 2013.
- [25] R. Fazel, H.M. Krumholz, Y. Wang, J.S. Ross, J. Chen, H.H. Ting *et al.*, "Exposure to low-dose ionizing radiation from medical imaging procedures," *New England Journal of Medicine* **361**(9), 849-857 (2009).

-
- [26] E.C. Lin, "Radiation risk from medical imaging," *Mayo Clinic Proceedings* **85**(12), 1142-1146 (2010).
 - [27] J. Hainfeld, D. Slatkin, T. Focella and H. Smilowitz, "Gold nanoparticles: A new X-ray contrast agent," *The British Institute of Radiology* **79**(939), 248-253 (2006).
 - [28] C. Müller and R. Schibli, "Single photon emission computed tomography tracer" In: *Molecular Imaging in Oncology*, Springer (Berlin) 2013.
 - [29] A. Granov, L. Tiutin and T. Schwarz, "The physical basis of positron emission tomography" In: *Positron Emission Tomography*, Springer (Berlin) 2013.
 - [30] S. Mallidi, G.P. Luke and S. Emelianov, "Photoacoustic imaging in cancer detection, diagnosis, and treatment guidance," *Trends in Biotechnology* **29**(5), 213-221 (2011).
 - [31] L.V. Wang and H. Wu, "Photoacoustic tomography" In: *Biomedical Optics: Principles and Imaging*, Wiley (New Jersey) 2007.
 - [32] V. Ntziachristos, C. Bremer and R. Weissleder, "Fluorescence imaging with near-infrared light: new technological advances that enable in vivo molecular imaging," *European Radiology* **13**(1), 195-208 (2003).
 - [33] K.-y. Ng and Y. Liu, "Therapeutic ultrasound: Its application in drug delivery," *Medicinal Research Reviews* **22**(2), 204-223 (2002).
 - [34] E.C. Unger, E. Hersh, M. Vannan and T. McCreery, "Gene delivery using ultrasound contrast agents," *Echocardiography* **18**(4), 355-361 (2001).
 - [35] S. Hu and L.V. Wang, "Photoacoustic imaging and characterization of the microvasculature," *Journal of Biomedical Optics* **15**(1), 011101 (2010).
 - [36] B.-Y. Hsieh, S.-L. Chen, T. Ling, L.J. Guo and P.-C. Li, "Integrated intravascular ultrasound and photoacoustic imaging scan head," *Optics Letters* **35**(17), 2892-2894 (2010).
 - [37] B.R. Benacerraf, C.B. Benson, A.Z. Abuhamad, J.A. Copel, J.S. Abramowicz, G.R. DeVore *et al.*, "Three- and 4-dimensional ultrasound in obstetrics and gynecology," *Journal of Ultrasound in Medicine* **24**(12), 1587-1597 (2005).
 - [38] P.V. Prasad, *Magnetic Resonance Imaging: Methods and Biologic Applications*, Humana Press (New York) 2006.
 - [39] K. Raymond, T.E. Romesser, J. Marmo and M.A. Folkman, "Airborne and satellite imaging spectrometer development at TRW," *Proc. SPIE* **2480**, 287-294 (1995).
-

-
- [40] J. Nieke, H.H. Schwarzer, A. Neumann and G. Zimmermann, "Imaging spaceborne and airborne sensor systems in the beginning of the next century," *Proc. SPIE* **3221**, 581-592 (1997).
- [41] G. Vaglio Laurin, J.C.-W. Chan, Q. Chen, J.A. Lindsell, D.A. Coomes, L. Guerriero *et al.*, "Biodiversity mapping in a tropical West African forest with airborne hyperspectral data," *PLoS ONE* **9**(6), e97910 (2014).
- [42] D. Lorente, N. Aleixos, J. Gómez-Sanchis, S. Cubero, O.L. García-Navarrete and J. Blasco, "Recent advances and applications of hyperspectral imaging for fruit and vegetable quality assessment," *Food and Bioprocess Technology* **5**(4), 1121-1142 (2012).
- [43] H. Feng, N. Jiang, C. Huang, W. Fang, W. Yang, G. Chen *et al.*, "A hyperspectral imaging system for an accurate prediction of the above-ground biomass of individual rice plants," *Review of Scientific Instruments* **84**(9), 095107 (2013).
- [44] K.M. O'Brien, J. Wren, V.K. Davé, D. Bai, R.D. Anderson, S. Rayner *et al.*, "ASTRAL, a hyperspectral imaging DNA sequencer," *Review of Scientific Instruments* **69**(5), 2141-2146 (1998).
- [45] E.B. Brauns and R.B. Dyer, "Fourier transform hyperspectral visible imaging and the nondestructive analysis of potentially fraudulent documents," *Applied Spectroscopy* **60**(8), 833-840 (2006).
- [46] S. Sumriddetchkajorn and Y. Intaravanne, "Hyperspectral imaging-based credit card verifier structure with adaptive learning," *Applied Optics* **47**(35), 6594-6600 (2008).
- [47] Z. Liu, S. Ma, Y. Ji, L. Liu, J. Guo and Y. He, "Parallel scan hyperspectral fluorescence imaging system and biomedical application for microarrays," *Journal of Physics: Conference Series* **277**(1), 012023 (2011).
- [48] V. Fresse, D. Houzet and C. Gravier, "GPU architecture evaluation for multispectral and hyperspectral image analysis," *Conference on Design and Architectures for Signal and Image Processing*, 121-127 (2010).
- [49] J.J. Puschell, "Hyperspectral imagers for current and future missions," *Proc. SPIE* **4041**, 121-132 (2000).
- [50] A.D. Meigs, L. Otten III and T.Y. Cherezova, "Ultraspectral imaging: A new contribution to global virtual presence," *Aerospace Conference* **2**, 5-12 (1998).
- [51] N. Gat, "Imaging spectroscopy using tunable filters: A review," *Proc. SPIE* **4056**, 50-64 (2000).
-

-
- [52] T. Arnold, M. De Biasio and R. Leitner, "High-sensitivity hyper-spectral video endoscopy system for intra-surgical tissue classification," *IEEE Sensors*, 2612-2615 (2010).
- [53] N. Hagen, R.T. Kester, L. Gao and T.S. Tkaczyk, "Snapshot advantage: A review of the light collection improvement for parallel high-dimensional measurement systems," *Optical Engineering* **51**(11), 111702 (2012).
- [54] M.B. Sinclair, D.M. Haaland, J.A. Timlin and H.D.T. Jones, "Hyperspectral confocal microscope," *Applied Optics* **45**(24), 6283-6291 (2006).
- [55] J.M. Medina, L.M. Pereira, H.T. Correia and S.M. Nascimento, "Hyperspectral optical imaging of human iris in vivo: characteristics of reflectance spectra," *Journal of Biomedical Optics* **16**(7), 076001 (2011).
- [56] J. Kriesel, G. Scriven, N. Gat, S. Nagaraj, P. Willson and V. Swaminathan, "Snapshot hyperspectral fovea vision system (HyperVideo)," *Proc. SPIE* **8390**, 83900T (2012).
- [57] M. Kosec, M. Bürmen, D. Tomažević, F. Pernuš and B. Likar, "Characterization of a spectrograph based hyperspectral imaging system," *Optics Express* **21**(10), 12085-12099 (2013).
- [58] M.B. Sinclair, J.A. Timlin, D.M. Haaland and M. Werner-Washburne, "Design, construction, characterization, and application of a hyperspectral microarray scanner," *Applied Optics* **43**(10), 2079-2088 (2004).
- [59] Y. Wang, S. Bish, J.W. Tunnell and X. Zhang, "MEMS scanner enabled real-time depth sensitive hyperspectral imaging of biological tissue," *Optics Express* **18**(23), 24101-24108 (2010).
- [60] R.A. Schultz, T. Nielsen, J.R. Zavaleta, R. Ruch, R. Wyatt and H.R. Garner, "Hyperspectral imaging: A novel approach for microscopic analysis," *Cytometry* **43**(4), 239-247 (2001).
- [61] F. Dell'Endice, J. Nieke, B. Koetz, M.E. Schaepman and K. Itten, "Improving radiometry of imaging spectrometers by using programmable spectral regions of interest," *ISPRS Journal of Photogrammetry and Remote Sensing* **64**(6), 632-639 (2009).
- [62] S. Kong, M. Martin and T. Vo-Dinh, "Hyperspectral fluorescence imaging for mouse skin tumor detection," *ETRI Journal* **28**(6), 770-776 (2006).
- [63] R. Leitner, T. Arnold and M. De Biasio, "High-sensitivity hyperspectral imager for biomedical video diagnostic applications," *Proc. SPIE* **7674**, 76740E (2010).
-

-
- [64] Y. Guan, Q. Li, H. Liu, L. Xu and Z. Zhu, "New-styled system based on hyperspectral imaging," *Symposium on Photonics and Optoelectronics*, 1-3 (2011).
- [65] M.E. Martin, M.B. Wabuyele, K. Chen, P. Kasili, M. Panjehpour, M. Phan *et al.*, "Development of an advanced hyperspectral imaging (HSI) system with applications for cancer detection," *Annals of Biomedical Engineering* **34**(6), 1061-1068 (2006).
- [66] B.S. Sorg, B.J. Moeller, O. Donovan, Y. Cao and M.W. Dewhirst, "Hyperspectral imaging of hemoglobin saturation in tumor microvasculature and tumor hypoxia development," *Journal of Biomedical Optics* **10**(4), 044004 (2005).
- [67] R. Leitner, M.D. Biasio, T. Arnold, C.V. Dinh, M. Loog and R.P. Duin, "Multi-spectral video endoscopy system for the detection of cancerous tissue," *Pattern Recognition Letters* **34**(1), 85-93 (2013).
- [68] W.R. Johnson, D.W. Wilson, W. Fink, M. Humayun and G. Bearman, "Snapshot hyperspectral imaging in ophthalmology," *Journal of Biomedical Optics* **12**(1), 014036 (2007).
- [69] M. Gehm, R. John, D. Brady, R. Willett and T. Schulz, "Single-shot compressive spectral imaging with a dual-disperser architecture," *Optics Express* **15**(21), 14013-14027 (2007).
- [70] G. Lu and B. Fei, "Medical hyperspectral imaging: A review," *Journal of Biomedical Optics* **19**(1), 010901 (2014).
- [71] D. Ren and J. Allington-Smith, "On the application of integral field unit design theory for imaging spectroscopy," *Publications of the Astronomical Society of the Pacific* **114**(798), 866-878 (2002).
- [72] L. Gao, R.T. Smith and T.S. Tkaczyk, "Snapshot hyperspectral retinal camera with the image mapping spectrometer (IMS)," *Biomedical Optics Express* **3**(1), 48-54 (2012).
- [73] D.W. Fletcher-Holmes and A.R. Harvey, "A snapshot foveal hyperspectral imager," *Proc. SPIE* **4816**, 407-414 (2002).
- [74] N. Gat, G. Scriven, J. Garman, M.D. Li and J. Zhang, "Development of four-dimensional imaging spectrometers (4D-IS)," *Proc. SPIE* **6302**, 63020M (2006).
- [75] C. Vanderriest, "Integral field spectroscopy with optical fibres," *Tridimensional Optical Spectroscopic Methods in Astrophysics* **71**, 209-218 (1995).
- [76] B. Khoobehi, A. Khoobehi and P. Fournier, "Snapshot hyperspectral imaging to measure oxygen saturation in the retina using fiber bundle and multi-slit spectrometer," *Proc. SPIE* **8229**, 82291E (2012).
-

- [77] R.T. Kester, L. Gao, N. Bedard and T.S. Tkaczyk, "Real-time hyperspectral endoscope for early cancer diagnostics," *Proc. SPIE* **7555**, 75550A (2010).
- [78] M.E. Martin, M.B. Wabuyele, M. Panjehpour, M.N. Phan, B.F. Overholt, R.C. DeNovo *et al.*, "Dual modality fluorescence and reflectance hyperspectral imaging: principle and applications," *Proc. SPIE* **5692**, 133-139 (2005).
- [79] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nature Medicine* **1**(1), 27-30 (1995).
- [80] B.R. Zetter, "Angiogenesis and tumor metastasis," *Annual Review of Medicine* **49**(1), 407-424 (1998).
- [81] D. Hanahan and R.A. Weinberg, "Hallmarks of cancer: The next generation," *Cell* **144**(5), 646-674 (2011).
- [82] B. Khoobehi, J.M. Beach and H. Kawano, "Hyperspectral imaging for measurement of oxygen saturation in the optic nerve head," *Investigative Ophthalmology & Visual Science* **45**(5), 1464-1472 (2004).
- [83] E.L. Larsen, L.L. Randeberg, E. Olstad, O.A. Haugen, A. Aksnes and L.O. Svaasand, "Hyperspectral imaging of atherosclerotic plaques in vitro," *Journal of Biomedical Optics* **16**(2), 026011 (2011).
- [84] D.M. Haaland, H.D. Jones, M.B. Sinclair, B. Carson, C. Branda, J.F. Poschet *et al.*, "Hyperspectral confocal fluorescence imaging of cells," *Proc. SPIE* **6765**, 676509 (2007).
- [85] M.R. Chatni, J. Xia, R. Sohn, K. Maslov, Z. Guo, Y. Zhang *et al.*, "Tumor glucose metabolism imaged in vivo in small animals with whole-body photoacoustic computed tomography," *Journal of Biomedical Optics* **17**(7), 076012 (2012).
- [86] J. Laufer, E. Zhang, G. Raivich and P. Beard, "Three-dimensional noninvasive imaging of the vasculature in the mouse brain using a high resolution photoacoustic scanner," *Applied Optics* **48**(10), D299-D306 (2009).
- [87] A.A. Kosterev, Y.A. Bakhirkin, R. Curl and F. Tittel, "Quartz-enhanced photoacoustic spectroscopy," *Optics Letters* **27**(21), 1902-1904 (2002).
- [88] T. Schmid, U. Panne, R. Niessner and C. Haisch, "Optical absorbance measurements of opaque liquids by pulsed laser photoacoustic spectroscopy," *Analytical Chemistry* **81**(6), 2403-2409 (2009).
- [89] Y. Villanueva, E. Hondebrink, W. Petersen and W. Steenbergen, "Photoacoustic measurement of the Grüneisen parameter using an integrating sphere," *Review of Scientific Instruments* **85**(7), 074904 (2014).

- [90] L.V. Wang, "Multiscale photoacoustic microscopy and computed tomography," *Nature Photonics* **3**(9), 503-509 (2009).
 - [91] L.V. Wang and S. Hu, "Photoacoustic tomography: In vivo imaging from organelles to organs," *Science* **335**(6075), 1458-1462 (2012).
 - [92] H.F. Zhang, K. Maslov, G. Stoica and L.V. Wang, "Functional photoacoustic microscopy for high-resolution and noninvasive in vivo imaging," *Nature Biotechnology* **24**(7), 848-851 (2006).
 - [93] J.J. Niederhauser, M. Jaeger, R. Lemor, P. Weber and M. Frenz, "Combined ultrasound and optoacoustic system for real-time high-contrast vascular imaging in vivo," *IEEE Transactions on Medical Imaging* **24**(4), 436-440 (2005).
 - [94] C. Zhang and Y. Wang, "Comparison of various imaging modes for photoacoustic tomography," *13th International Conference on Biomedical Engineering*, 121-124 (2009).
 - [95] C. Zhang, K. Maslov and L.V. Wang, "Subwavelength-resolution label-free photoacoustic microscopy of optical absorption in vivo," *Optics Letters* **35**(19), 3195-3197 (2010).
 - [96] S. Hu, K. Maslov and L.V. Wang, "Second-generation optical-resolution photoacoustic microscopy with improved sensitivity and speed," *Optics Letters* **36**(7), 1134-1136 (2011).
 - [97] K. Maslov, H.F. Zhang, S. Hu and L.V. Wang, "Optical-resolution photoacoustic microscopy for in vivo imaging of single capillaries," *Optics Letters* **33**(9), 929-931 (2008).
 - [98] M. Xu and L.V. Wang, "Time-domain reconstruction for thermoacoustic tomography in a spherical geometry," *IEEE Transactions on Medical Imaging* **21**(7), 814-822 (2002).
 - [99] J.-M. Yang, K. Maslov, H.-C. Yang, Q. Zhou, K.K. Shung and L.V. Wang, "Photoacoustic endoscopy," *Optics Letters* **34**(10), 1591-1593 (2009).
 - [100] Y. Yuan, S. Yang and D. Xing, "Preclinical photoacoustic imaging endoscope based on acousto-optic coaxial system using ring transducer array," *Optics Letters* **35**(13), 2266-2268 (2010).
 - [101] Z. Xu, C. Li and L.V. Wang, "Photoacoustic tomography of water in phantoms and tissue," *Journal of Biomedical Optics* **15**(3), 036019 (2010).
 - [102] P.M. Morse and K.U. Ingard, *Theoretical Acoustics*, McGraw-Hill (New York) 1968.
-

-
- [103] L.V. Wang, "Tutorial on photoacoustic microscopy and computed tomography," *IEEE Journal of Selected Topics in Quantum Electronics* **14**(1), 171-179 (2008).
- [104] G.P. Luke, S.Y. Nam and S.Y. Emelianov, "Optical wavelength selection for improved spectroscopic photoacoustic imaging," *Photoacoustics* **1**(2), 36-42 (2013).
- [105] J. Laufer, A. Jathoul, M. Pule and P. Beard, "In vitro characterization of genetically expressed absorbing proteins using photoacoustic spectroscopy," *Biomedical Optics Express* **4**(11), 2477-2490 (2013).
- [106] D.-K. Yao, C. Zhang, K. Maslov and L.V. Wang, "Photoacoustic measurement of the Grüneisen parameter of tissue," *Journal of Biomedical Optics* **19**(1), 017007 (2014).
- [107] L. Yu and V. Giurgiutiu, "Advanced signal processing for enhanced damage detection with piezoelectric wafer active sensors," *Smart Structures and Systems* **1**(2), 185-215 (2005).
- [108] B. Wang, E. Yantsen, T. Larson, A.B. Karpiouk, S. Sethuraman, J.L. Su *et al.*, "Plasmonic intravascular photoacoustic imaging for detection of macrophages in atherosclerotic plaques," *Nano Letters* **9**(6), 2212-2217 (2008).
- [109] J.-M. Yang, C. Favazza, R. Chen, J. Yao, X. Cai, K. Maslov *et al.*, "Simultaneous functional photoacoustic and ultrasonic endoscopy of internal organs in vivo," *Nature Medicine* **18**(8), 1297-1302 (2012).
- [110] M. Li, J. Oh, X. Xie, G. Ku, W. Wang, C. Li *et al.*, "Simultaneous molecular and hypoxia imaging of brain tumors in vivo using spectroscopic photoacoustic tomography," *Proceedings of the IEEE* **96**(3), 481 (2008).
- [111] Y.N. Billeh, M. Liu and T. Buma, "Spectroscopic photoacoustic microscopy using a photonic crystal fiber supercontinuum source," *Optics Express* **18**(18), 18519-18524 (2010).
- [112] D. Razansky, M. Distel, C. Vinegoni, R. Ma, N. Perrimon, R.W. Köster *et al.*, "Multispectral opto-acoustic tomography of deep-seated fluorescent proteins in vivo," *Nature Photonics* **3**(7), 412-417 (2009).
- [113] S. Sethuraman, J.H. Amirian, S.H. Litovsky, R.W. Smalling and S.Y. Emelianov, "Spectroscopic intravascular photoacoustic imaging to differentiate atherosclerotic plaques," *Optics Express* **16**(5), 3362-3367 (2008).
- [114] B. Wang, A. Karpiouk, D. Yeager, J. Amirian, S. Litovsky, R. Smalling *et al.*, "Intravascular photoacoustic imaging of lipid in atherosclerotic plaques in the presence of luminal blood," *Optics Letters* **37**(7), 1244-1246 (2012).
-

- [115] L.H. Arroyo and R.T. Lee, "Mechanisms of plaque rupture: Mechanical and biologic interactions," *Cardiovascular Research* **41**(2), 369-375 (1999).
 - [116] N. Lewinski, V. Colvin and R. Drezek, "Cytotoxicity of nanoparticles," *Small* **4**(1), 26-49 (2008).
 - [117] S.K. Maji, S. Sreejith, J. Joseph, M. Lin, T. He, Y. Tong *et al.*, "Upconversion nanoparticles as a contrast agent for photoacoustic imaging in live mice," *Advanced Materials* **26**(32), 5633-5638 (2014).
 - [118] X. Yang, E.W. Stein, S. Ashkenazi and L.V. Wang, "Nanoparticles for photoacoustic imaging," *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* **1**(4), 360-368 (2009).
 - [119] D.A. Schultz, "Plasmon resonant particles for biological detection," *Current Opinion in Biotechnology* **14**(1), 13-22 (2003).
 - [120] M.L. Li, J.C. Wang, J.A. Schwartz, K.L. Gill-Sharp, G. Stoica and L.V. Wang, "In-vivo photoacoustic microscopy of nanoshell extravasation from solid tumor vasculature," *Journal of Biomedical Optics* **14**(1), 010507 (2009).
 - [121] A. Lin, L. Hirsch, M.H. Lee, J. Barton, N. Halas, J. West *et al.*, "Nanoshell-enabled photonics-based imaging and therapy of cancer," *Technology in Cancer Research & Treatment* **3**(1), 33-40 (2004).
 - [122] J. Becker, A. Trügler, A. Jakab, U. Hohenester and C. Sönnichsen, "The optimal aspect ratio of gold nanorods for plasmonic bio-sensing," *Plasmonics* **5**(2), 161-167 (2010).
 - [123] D. Razansky, C. Vinegoni and V. Ntziachristos, "Multispectral photoacoustic imaging of fluorochromes in small animals," *Optics Letters* **32**(19), 2891-2893 (2007).
 - [124] G.M. Palmer, P.J. Keely, T.M. Breslin and N. Ramanujam, "Autofluorescence spectroscopy of normal and malignant human breast cell lines," *Photochemistry and Photobiology* **78**(5), 462-469 (2003).
 - [125] N. Ramanujam, "Fluorescence spectroscopy in vivo" In: *Encyclopedia of Analytical Chemistry*, Wiley (New Jersey) 2000.
 - [126] B.-H. Li and S.-S. Xie, "Autofluorescence excitation-emission matrices for diagnosis of colonic cancer," *World Journal of Gastroenterology* **11**(25), 3931-3934 (2005).
 - [127] Z. Liu, H. Shi, L. Liu, S. Deng, Y. Ji, S. Ma *et al.*, "Line-monitoring, hyperspectral fluorescence setup for simultaneous multi-analyte biosensing," *Sensors* **11**(11), 10038-10047 (2011).
-

-
- [128] J.W. Uhr, M.L. Huebschman, E.P. Frenkel, N.L. Lane, R. Ashfaq, H. Liu *et al.*, "Molecular profiling of individual tumor cells by hyperspectral microscopic imaging," *Translational Research* **159**(5), 366-375 (2012).
 - [129] S.C. Barden and R.A. Wade, "DensePak and spectral imaging with fiber optics," *Fiber Optics in Astronomy* **3**, 113-124 (1988).
 - [130] M. Sun, D. Zhang, Z. Wang, J. Ren, B. Chai and J. Sun, "What's wrong with the murals at the Mogao Grottoes: A near-infrared hyperspectral imaging method," *Scientific Reports* **5**, 14371 (2015).
 - [131] W. Jahr, B. Schmid, C. Schmied, F.O. Fahrbach and J. Huisken, "Hyperspectral light sheet microscopy," *Nature Communications* **6**, 7990 (2015).
 - [132] F. Vasefi, N. MacKinnon, R.B. Saager, A.J. Durkin, R. Chave, E.H. Lindsley *et al.*, "Polarization-sensitive hyperspectral imaging in vivo: A multimode dermoscope for skin analysis," *Scientific Reports* **4**, 4924 (2014).
 - [133] M.P. Nelson and M.L. Myrick, "Fabrication and evaluation of a dimension-reduction fiberoptic system for chemical imaging applications," *Review of Scientific Instruments* **70**(6), 2836-2844 (1999).
 - [134] C.A. Massaad, G. Zhang, L. Pillai, A. Azhdarinia, W. Liu and K.A. Sheikh, "Fluorescently-tagged anti-ganglioside antibody selectively identifies peripheral nerve in living animals," *Scientific Reports* **5**, 15766 (2015).
 - [135] A.D. Mehta, J.C. Jung, B.A. Flusberg and M.J. Schnitzer, "Fiber optic in vivo imaging in the mammalian nervous system," *Current Opinion in Neurobiology* **14**(5), 617-628 (2004).
 - [136] A. Klimas and E. Entcheva, "Toward microendoscopy-inspired cardiac optogenetics in vivo: Technical overview and perspective," *Journal of Biomedical Optics* **19**(8), 080701 (2014).
 - [137] H.A. Quigley and A.T. Broman, "The number of people with glaucoma worldwide in 2010 and 2020," *British Journal of Ophthalmology* **90**(3), 262-267 (2006).
 - [138] S. Hu, B. Rao, K. Maslov and L.V. Wang, "Label-free photoacoustic ophthalmic angiography," *Optics Letters* **35**(1), 1-3 (2010).
 - [139] H. Estrada, E. Sobol, O. Baum and D. Razansky, "Hybrid optoacoustic and ultrasound biomicroscopy monitors' laser-induced tissue modifications and magnetite nanoparticle impregnation," *Laser Physics Letters* **11**(12), 125601 (2014).
 - [140] P. Wang, P. Wang, H.-W. Wang and J.-X. Cheng, "Hyperspectral vibrational photoacoustic imaging of lipids and collagen," *Proc. SPIE* **8223**, 82231I (2012).
-

- [141] Y. Shen, Z. Lu, S. Spiers, H.A. MacKenzie, H.S. Ashton, J. Hannigan *et al.*, "Measurement of the optical absorption coefficient of a liquid by use of a time-resolved photoacoustic technique," *Applied Optics* **39**(22), 4007-4012 (2000).
- [142] R. Fuente, E. Apinaniz, A. Mendioroz and A. Salazar, "Simultaneous measurement of thermal diffusivity and optical absorption coefficient using photothermal radiometry. I. Homogeneous solids," *Journal of Applied Physics* **110**(3), 033515 (2011).
- [143] W.P. Arnott, H. Moosmüller, C.F. Rogers, T. Jin and R. Bruch, "Photoacoustic spectrometer for measuring light absorption by aerosol: Instrument description," *Atmospheric Environment* **33**(17), 2845-2852 (1999).
- [144] A. de La Zerda, Y.M. Paulus, R. Teed, S. Bodapati, Y. Dollberg, B.T. Khuri-Yakub *et al.*, "Photoacoustic ocular imaging," *Optics Letters* **35**(3), 270-272 (2010).
- [145] J. Xia, E. Berg, J. Lee and G. Yao, "Characterizing beef muscles with optical scattering and absorption coefficients in VIS-NIR region," *Meat Science* **75**(1), 78-83 (2007).
- [146] G. Marquez, L.V. Wang, S.-P. Lin, J.A. Schwartz and S.L. Thomsen, "Anisotropy in the absorption and scattering spectra of chicken breast tissue," *Applied Optics* **37**(4), 798-804 (1998).
- [147] L. Wang and S.L. Jacques, "Use of a laser beam with an oblique angle of incidence to measure the reduced scattering coefficient of a turbid medium," *Applied Optics* **34**(13), 2362-2366 (1995).
- [148] International Commission on Non-Ionizing Radiation Protection, "ICNIRP guidelines on limits of exposure to laser radiation of wavelengths between 180 nm and 1,000 μm ," *Health Physics* **105**(3), 271-295 (2013).
- [149] J. Ruiz-Ederra, M. García, M. Hernández, H. Urcola, E. Hernández-Barbáchano, J. Araiz *et al.*, "The pig eye as a novel model of glaucoma," *Experimental Eye Research* **81**(5), 561-569 (2005).
- [150] C. Faber, M. Wang, E. Scherfig, K.E. Sørensen, J.U. Prause, N. Ehlers *et al.*, "Orthotopic porcine corneal xenotransplantation using a human graft," *Acta Ophthalmologica* **87**(8), 917-919 (2009).
- [151] L. Jay, A. Brocas, K. Singh, J.-C. Kieffer, I. Brunette and T. Ozaki, "Determination of porcine corneal layers with high spatial resolution by simultaneous second and third harmonic generation microscopy," *Optics Express* **16**(21), 16284-16293 (2008).

-
- [152] S.E. Skrabalak, J. Chen, Y. Sun, X. Lu, L. Au, C.M. Cobley *et al.*, “Gold nanocages: Synthesis, properties, and applications,” *Accounts of Chemical Research* **41**(12), 1587-1595 (2008).
- [153] Y. Wang, K.C.L. Black, H. Luehmann, W. Li, Y. Zhang, X. Cai *et al.*, “Comparison study of gold nanoheptapods, nanorods, and nanocages for photothermal cancer treatment,” *ACS Nano* **7**(3), 2068-2077 (2013).
- [154] J. Chen, J.M. McLellan, A. Siekkinen, Y. Xiong, Z.-Y. Li and Y. Xia, “Facile synthesis of gold–silver nanocages with controllable pores on the surface,” *Journal of the American Chemical Society* **128**(46), 14776-14777 (2006).
- [155] S.E. Skrabalak, L. Au, X. Li and Y. Xia, “Facile synthesis of Ag nanocubes and Au nanocages,” *Nature Protocols* **2**(9), 2182-2190 (2007).
- [156] C. Kim, E.C. Cho, J. Chen, K.H. Song, L. Au, C. Favazza *et al.*, “In vivo molecular photoacoustic tomography of melanomas targeted by bioconjugated gold nanocages,” *ACS Nano* **4**(8), 4559-4564 (2010).
- [157] X. Yang, S.E. Skrabalak, Z.-Y. Li, Y. Xia and L.V. Wang, “Photoacoustic tomography of a rat cerebral cortex in vivo with Au nanocages as an optical contrast agent,” *Nano Letters* **7**(12), 3798-3802 (2007).
- [158] J. Chen, F. Saeki, B.J. Wiley, H. Cang, M.J. Cobb, Z.-Y. Li *et al.*, “Gold nanocages: Bioconjugation and their potential use as optical imaging contrast agents,” *Nano Letters* **5**(3), 473-477 (2005).
- [159] W. Song, Q. Wei, W. Liu, T. Liu, J. Yi, N. Sheibani *et al.*, “A combined method to quantify the retinal metabolic rate of oxygen using photoacoustic ophthalmoscopy and optical coherence tomography,” *Scientific Reports* **4**, 6525 (2014).
- [160] D. Chen, X. Qiao, X. Qiu, J. Chen and R. Jiang, “Convenient, rapid synthesis of silver nanocubes and nanowires via a microwave-assisted polyol method,” *Nanotechnology* **21**(2), 025607 (2010).
- [161] S. Liu, X. Zheng, L. Song, W. Liu, T. Yao, Z. Sun *et al.*, “Partial-surface-passivation strategy for transition-metal-based copper-gold nanocage,” *Chemical Communications* **52**(39), 6617-6620 (2016).
-