Probe-based confocal laser endomicroscopy: an evaluation of its role towards real-time, \textit{in vivo, in situ} intraoperative applications

Tou Pin Chang

MB ChB, BSc (Hons), MRCS

Supervisors:

Professor The Lord Ara Darzi of Denham

Professor Guang-Zhong Yang

The Hamlyn Centre for Robotic Surgery,

Institute of Global Health Innovation,

Imperial College London

Doctor of Philosophy (PhD)

2016
This thesis is submitted to the Imperial College London in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Except for where specifically described in the acknowledgements section, it presents entirely my own work and describes the results of my own research.

The copyright rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution researchers must make clear to others the licence terms of this work.
Abstract

Probe-based confocal laser endomicroscopy (pCLE) is an emerging imaging tool that allows real-time in situ morphological imaging at cellular and subcellular resolution. Its ability to image morphological features of epithelial surfaces of the gastrointestinal tract, biliary tree and respiratory tree rendered differentiation of macroscopically inconspicuous neoplastic and non-neoplastic tissues possible in real-time. However, its role outwith the endoluminal environment for surgical applications has been comparatively sparsely investigated and little reported on its ability to characterise morphological features beyond endoluminal applications. This thesis aims to systematically evaluate the potential pCLE has in visualization of soft tissue morphology in applications pertaining to breast conserving surgery (BCS), parathyroid surgery and thyroid surgery; whereby morphological information regarding cavity wall margin status, tissue-specific entity and viability status of preserved parathyroid glands (PG), respectively, could potentially guide decision-making intraoperatively.

The perceptions that pCLE imaging is confined to endoluminal mucosal surfaces, the inability of pCLE to perform image acquisition through sterile transparent sheaths and the inability of surgeons to interpret pCLE images were interrogated using three small feasibility studies. Firstly, in a study carried out on a live, anaesthetised, porcine model, pCLE image acquisition of morphological architecture of soft tissues of the neck e.g. thyroid, lymph nodes, adipose, skeletal and smooth muscles, were shown to be feasible in an intraoperative field and the presence dried blood on the tissue surface did not impede the consistency of morphological architecture visualization. Secondly, we demonstrated that utilization of a sterile transparent sheath did not impede pCLE image acquisition and that the quality of images obtained was comparable to that of without the sheath. Thirdly, we have shown that surgeons with little or no histopathology background were able to acquire the relevant pattern recognition skills to interpret pCLE images following a training session utilizing a validated pCLE morphological classification from colorectal lesions.

Building upon these discoveries, we elucidated the potential of pCLE to image neoplastic and non-neoplastic breast morphology with the envisaged application of identifying residual cancerous foci
intraoperatively, thereby guiding operative decision making based on real-time breast cavity scanning during BCS. Preliminary ex vivo analyses from 71 freshly excised, acriflavine-stained neoplastic and non-neoplastic tissues samples from 50 breast cancer patients show excellent correlation with histopathology findings. In particular, the glandular structures, adipocytes and collagen fibres of non-neoplastic breast tissues were readily visualised on pCLE images. These were distinguishable from the markedly haphazard and hypercellular architecture exhibited by invasive and non-invasive carcinoma. We developed a classification based on description of pCLE morphology unique to neoplastic and non-neoplastic breast morphology and validated this with 17 histopathologists and surgeons through a systematic pattern recognition training session based on this classification where they were subsequently subjected to objective assessment of 50 pCLE images while blinded to histopathology results. The overall mean accuracy of pCLE image interpretation for histopathologists and surgeons were 94% and 92%, respectively. The overall inter-observer agreement was ‘almost perfect’ (κ=0.81) for the former and ‘substantial’ (κ=0.77), for the latter.

We explored the role of intravenous fluorescein sodium (FS) in a prospective, cross-sectional, observational study of 10 patients undergoing BCS where they received between 1.5ml to 3.5ml of intravenous bolus of 10% fluorescein sodium (FS) intraoperatively. Ex vivo analyses of FS-stained breast samples showed that dense fibrous tissue response evoked by infiltrating tumor cells were readily visualised as fluorescent regions with haphazardly arranged, amorphous-looking collagen fibres. However, the lack of nuclei visualization rendered differentiation of neoplastic from non-neoplastic tissues impossible. Nevertheless, the uniformity that FS staining imparts to all tissue layers facilitated creation of longer and meaningful pCLE mosaics. These findings could have important implications where tissue deformation could result in AH-stained layers intermittently fail to coincide with the optical slice imaged at the respective depth.

The promising findings of AH-stained breast tissues were found to be potentially relevant in parathyroid surgery. Similar analyses on freshly excised AH-stained parathyroid specimens from 35 patients undergoing parathyroidectomy for primary and secondary hyperparathyroidism showed nest-like arrangements of parenchymal cells, fibrovascular septum and microfollicles of diseased PGs were
readily identifiable on CE images and these were consistent with histopathological findings. Following pattern recognition training based on an in-house developed classification system, these were distinguishable from epithelial-lined thyroid follicles and polygonal-shaped adipocytes with mean accuracies of 94% and 93% for histopathologists and surgeons, respectively, and high overall inter-observer agreement, $\kappa=0.82$. Where intraoperative identification of diseased PGs presents a challenge especially in multi-glandular disease and re-operative surgery, pCLE could potentially facilitate its recognition.

Finally, the role for pCLE imaging of PG vasculature was explored by means of an intraoperative clinical study utilising a sterile-transparent draped pCLE probe on 20 patients undergoing thyroid and parathyroid surgery. Utilising intravenous FS, branched-vessels including capillary networks were readily visualised. Vascular flow on viable glands was depicted by unidirectional, high velocity thrusts of dark-coloured erythrocytes within hyperfluorescent vessels or diffusely in the parenchyma whereas these were absent on non-viable (post-excision) glands. Further analysis on preserved PGs showed that absence of blood flow was found in patients who had sub-optimal post-operative parathyroid function. Given that visual assessment of tissue discolouration is not a reliable method of determining parathyroid gland viability during thyroidectomy, information regarding viability of preserved PGs decisions could potentially aid decisions pertinent to autotransplantation remains challenging.

This thesis significantly expands upon the potential intraoperative applications of pCLE. Evidently, these findings are preliminary and warrant further evaluation in well-powered clinical trials. However a systematic approach to investigate the optimal trade-offs between the optical resolution requirements of tissue morphology visualization and deployability of pCLE probe holds the key to successful clinical translation. In particular, evaluation of a robust mechatronically enhanced platform equipped with the flexibility to cater for tissue surface deformation and precision mechanisms that generates accurate spatio-temporal localisation in real-time to aid intraoperative decision making constitutes the next stage of research priorities.
Acknowledgements

I am most grateful to my two supervisors, Professor Lord Ara Darzi and Professor Guang-Zhong Yang, who supported me throughout my journey in obtaining this PhD. Professor Darzi inspired me with his tremendous enthusiasm, endless hunger and drive to deliver innovative technological solutions to challenges faced during surgery. Professor Yang demonstrated endless energy, encyclopaedic knowledge of imaging technologies and has offered me encouragement and emotional support in times of difficulty, for which I am very grateful for. Both possess broad knowledge on the subject, diverse academic experience and steadfast interests which are admirable aids for a PhD student to have. Both always encouraged me to realise my full potential in research by taking pride in my work and in myself. Both taught me many essential skills in research, but more importantly they showed me the way to think critically, persevere and think laterally, which have been instrumental in my development as an independent and mature academic.

I shall forever remember the support and generosity I received from my project collaborators from Charing Cross and Hammersmith Hospitals which includes Professor Sami Shousha, Dr Rathi Ramakrishnan, Dr Rashpal Flora, Dr Mihir Gudi and the Histopathology Department; Mr Dimitri Hadjiminas, Mr Ragheed Al-Mufti and Imperial Breast Unit; Mr Fausto Palazzo, Mr Neil Tolley, Mr Vasilis Constantinides and Department of Endocrine and Thyroid Surgery. I am also very grateful to Mr Daniel Leff, who saw a potential in me at a time when no one else did. He had faith in me and dedicated personal time to assist me with some of the projects and my professional development.

I would also like to thank my Hamlyn Centre colleagues Dr Michael Hughes, Dr Siyang Zuo, Mr Petros Giataganas, Dr Jianzhong Shang, Dr Win Tun Latt and Dr Ka-Wai Kwok for the multitude of insightful discussions that enhanced my knowledge and appreciation on the breadth of technical challenges that needs to be considered when formulating engineering solutions to address clinical problems. My fellow surgical trainee colleagues Mr Kumuthan Sriskandarajah, Mr Muzzafer
Chaudery, Mr Kunal Shetty, Mr Tom Cundy, Mr Myutan Kulendran, Mr Jochem Caris and Mr Richard Newton have also been valuable resources and have helped make the whole PhD experience thoroughly enjoyable.

I would like to thank the National Institute of Healthcare Research (NIHR) Imperial Biomedical Research Council for their project grant on laboratory consumables. I am grateful to all the patients, surgical colleagues and histopathologists who took part in these studies and for being so willing and generous.

I should also like to thank the following:

- Ms Cathy Gray and Ms Farhana Surti for research assistance during porcine studies at Northwick Park Institute of Medical Research.
- Dr Kevin Leary for immunohistochemistry and general histology advice.
- Mr Chetabhan Patel, Ms Vishni Ujoodni and Ms Amira Ibrahim for technical support with tissue sample preparations
- Mr John Nicklin for general administration and e-mail updates about research participants.
- Mr Didier Laure and Ms Alexandra Schmidt for technical support with the pCLE machine and image interpretation

Above all, I would like to thank The Hamlyn Centre, Raphaelle Raupp, Ruzanna Gulakyan and Karen Kerr for administrative and facility support.

Finally, I extend my gratitude to my wife, Elena; the most supportive, beautiful, loving and understanding person I have ever known. Thank you for your patience.
To my mum, Tou Chen, Tou Li, Elena

and Baby Alexander Shang Ren Chang.....

I am here because you were there
Contents

Abstract

Acknowledgements

Contents

List of Figures .................................................................................................................. i

List of Tables: .................................................................................................................. xii

List of Acronyms: .......................................................................................................... xiv

Chapter 1: Introduction and Overview ........................................................................ 1

1.1 Introduction ............................................................................................................ 1

1.2 Thesis chapter synopsis ....................................................................................... 2

1.3 The main scientific contributions in this thesis ................................................... 6

1.4 Prizes and publications associated with this thesis .............................................. 7

Chapter 2: Overview of probe-based confocal laser endomicroscopy ...................... 11

2.1 Evolution of probe-based confocal laser endomicroscopy ................................ 11

2.1.1 Principles of confocal microscopy .................................................................. 11

2.1.2 Confocal laser endomicroscopy ................................................................... 15

2.1.3 Contrast agents for pCLE imaging ............................................................... 20

2.2 Overview of the potential clinical applications for pCLE ................................ 23

2.2.1 Overview ....................................................................................................... 23
2.2.2 pCLE imaging in the upper gastrointestinal tract .............................................. 23
2.2.3 pCLE imaging in the lower gastrointestinal tract .............................................. 26
2.2.4 pCLE imaging in the biliary tree ........................................................................ 29
2.2.5 pCLE imaging in the urinary tract ..................................................................... 32
2.2.6 pCLE (nCLE) imaging of pancreatic lesions .................................................... 34
2.2.7 pCLE imaging of the respiratory tract ............................................................... 35
2.3 Challenges associated with pCLE imaging .......................................................... 36
2.3.1 Clinical challenges with envisaged applications ............................................... 36
2.3.2 pCLE image interpretation and miniprobe stability ......................................... 40
2.4 Role of pCLE imaging in surgery ......................................................................... 41
2.4.1 Overview ........................................................................................................... 41
2.4.2 Potential applications in breast and endocrine surgery .................................... 44
2.4.3 pCLE imaging of blood-stained tissues during surgery .................................... 49
2.4.4 pCLE imaging through a sterile transparent sheath ......................................... 52
2.4.5 pCLE image interpretation by surgeons ......................................................... 54

Chapter 3: Evaluation of Acriflavine-stained Breast Morphology on pCLE Images ........ 57
3.1 Introduction ........................................................................................................... 57
3.1.1 Positive margins in breast conserving surgery ................................................. 57
3.1.2 Challenges with margin assessment ................................................................. 57
3.1.3 Potential role for pCLE .................................................................................. 59
3.1.4 Aims and hypothesis ................................................. 59

3.2 Methods ................................................................. 60

3.2.1 Patients and tissue preparation ................................. 60

3.2.2 Equipment and image acquisition ............................. 60

3.2.3 Histopathology ..................................................... 63

3.2.4 Correlation of image mosaics ................................. 63

3.2.5 Image interpretation assessment ............................. 63

3.2.6 Statistical analysis ................................................. 64

3.3 Results ................................................................. 65

3.3.1 Acriflavine hydrochloride dose ............................... 65

3.3.2 pCLE image mosaics ............................................. 65

3.3.3 Correlation of pCLE image mosaics with histology (non-neoplastic) ......................... 65

3.3.4 Correlation of pCLE image mosaics with histology (neoplastic) ...................... 68

3.3.5 pCLE classification for breast morphology .................. 72

3.3.6 pCLE image interpretation assessment ..................... 76

3.4 Discussion ............................................................ 78

3.4.1 Understanding pCLE breast morphology .................... 78

3.4.2 pCLE breast classification .................................... 79

3.4.3 pCLE image interpretation – a surgical armamentarium? ......................... 80

3.4.4 Going beyond the endoluminal tract ........................... 82
3.4.5 Envisaged optical requirements ......................................................... 82
3.4.6 Acute inflammatory response ............................................................ 83
3.4.7 Benign breast conditions ................................................................. 84
3.5 Conclusion ......................................................................................... 85
Chapter 4: Evaluation of Fluorescein-stained Breast Morphology on pCLE Images ........ 86
4.1 Introduction ......................................................................................... 86
  4.1.1 Overview ....................................................................................... 86
  4.1.2 Hypothesis .................................................................................... 87
  4.1.3 Study design rationale ................................................................. 87
  4.1.4 Study aims ................................................................................... 88
4.2 Methods .............................................................................................. 88
  4.2.1 Patients ......................................................................................... 88
  4.2.2 Intravenous fluorescein ................................................................. 88
  4.2.3 Specimen handling ....................................................................... 89
  4.2.4 Image acquisition and equipment ................................................ 90
  4.2.5 Correlation with histology ........................................................... 90
  4.2.6 Evaluation of pCLE mosaic length ............................................... 90
  4.2.7 Statistical analysis ...................................................................... 91
4.3 Results ............................................................................................... 92
  4.3.1 Baseline characteristics ............................................................... 92
4.3.2 Correlation with histology ................................................................. 93
4.3.3 Length of pCLE mosaics................................................................. 98
4.4 Discussion ......................................................................................... 99
  4.4.1 Pathological fibrosis ................................................................. 99
  4.4.2 Potential pitfalls of image interpretation .................................. 99
  4.4.3 Envisaged benefits of FS-staining ........................................... 100
  4.4.4 FS-staining in the algorithm of cavity scanning ...................... 101
  4.4.5 Study limitations ................................................................. 102
  4.4.6 Conclusion ............................................................................. 103

Chapter 5: Evaluation of Acriflavine-stained Parathyroid Morphology on pCLE Images .......... 104

  5.1 Introduction .............................................................................. 104
    5.1.1 Minimally invasive parathyroidectomy .............................. 104
    5.1.2 Challenges with inconclusive localization tests ............... 104
    5.1.3 Potential role for pCLE ....................................................... 105
    5.1.4 Aims and hypothesis .......................................................... 106

  5.2 Methods .................................................................................... 106
    5.2.1 Patients and tissue preparation ........................................ 106
    5.2.2 Equipment and image acquisition .................................... 107
    5.2.3 Histopathology ................................................................. 107
    5.2.4 Correlation of image mosaics ........................................... 108
5.2.5 Image interpretation assessment .................................................. 109

5.2.6 Statistical analysis ........................................................................ 109

5.3 Results ................................................................................................. 110

5.3.1 Baseline pCLE image mosaic characteristics ........................................ 110

5.3.2 Correlation of pCLE image mosaics with histopathology ......................... 111

5.3.3 Accuracy of pCLE image interpretations .............................................. 120

5.3.4 Level of difficulty of pCLE image interpretation ..................................... 122

5.4 Discussion ............................................................................................... 125

5.4.1 Pattern recognition ............................................................................. 126

5.4.2 Image interpretation .......................................................................... 127

5.4.3 Intraoperative deployment ................................................................. 128

5.4.4 Potential intraoperative challenges .................................................... 129

5.4.5 Study limitations .............................................................................. 130

5.4.6 Conclusion .......................................................................................... 130

Chapter 6: Evaluation of Vascular Morphology of Fluorescein-Stained Parathyroid Glands on pCLE Images ................................................................. 132

6.1 Introduction ............................................................................................ 132

6.1.1 Postoperative hypoparathyroidism .................................................... 132

6.1.2 Challenges with PG viability assessment ............................................. 132

6.1.3 Vascular morphology on pCLE imaging ............................................ 134
6.1.4 Aims and hypothesis ................................................................. 134

6.2 Methods ....................................................................................... 135

6.2.1 Patients ...................................................................................... 135

6.2.2 Equipment .................................................................................. 135

6.2.3 Intraoperative pCLE image acquisition ....................................... 136

6.2.4 pCLE imaging for parathyroidectomy ......................................... 137

6.2.5 pCLE imaging for thyroidectomy ................................................ 137

6.2.6 Assessment of pCLE vascular morphology ................................. 137

6.2.7 Comparison with post-operative PTH and calcium levels .............. 139

6.2.8 Statistical analysis ....................................................................... 140

6.3 Results .......................................................................................... 140

6.3.1 Baseline characteristics .............................................................. 140

6.3.2 Vascular morphology description ............................................... 141

6.3.3 Vascular morphology evaluation ................................................ 147

6.3.4 Comparison with post-operative PTH function ............................. 148

6.4 Discussion ..................................................................................... 151

6.4.1 Overview ..................................................................................... 151

6.4.2 Visualization of vascular morphology ......................................... 151

6.4.3 Vessel recognition ....................................................................... 152

6.4.4 Vessel flow and flow rate .......................................................... 153
List of figures:

**Figure 1.1** The experimental chapters develop from four three ‘core surgical themes’ highlighted in Chapter 2.................................................................................................................................................... 5

**Figure 2.1** Optical pathways of tissues on conventional white light microscopy and confocal microscopy. (A) The clarity of the image produced by white light microscopy emanated from the pathways created from a thinly sliced sample as no other ‘out of focus’ pathways were created. (B) Confocal microscope illuminates a thick block of sample but only allow pathways created from that optical slice (confocal slice) to be filtered through the pinhole, hence eliminating all other out of focused pathways........................................................................................................................................... 13

**Figure 2.2** An example of confocal microscope (reflectance microscope) being used to perform real-time imaging on excised parathyroid specimens [Adapted from White et al, (X)] ……………… 15

**Figure 2.3** (A) Endoscope-based confocal endomicroscopy (eCLE) – Endomicroscope integrated at the distal tip of the endoscope (blue arrow). © 2012, Pentax (B) pCLE Cellvizio stack consisting of a personal computer display (top deck), computer processing unit (middle deck) and a laser scanning unit (lower deck). (C) pCLE miniprobe at the point of entry into a working channel of an endoscope. (D) pCLE miniprobe at the distal end of an endoscope with the distal tip of confocal miniprobe abutting the surface of gastrointestinal mucosa during endoscopy (insert) [Adapted from Chang et al, (X)] ……………………………………………………………………………………………………………. 16

**Figure 2.4** Images depicting the relative sizes of pCLE miniprobes. (A) The Cholangioflex® probe (left) is used for visualization of biliary strictures and the GastroflexUHD® probe (right) is deployed for upper gastrointestinal tract imaging. (B) Mini-O® pCLE miniprobe, a pre-clinical pCLE miniprobe with similar physical characteristics with a detachable plastic holder to facilitate stable apposition against tissue surfaces........................................................................................................................................... 17
Figure 2.5 Orientation of pCLE image acquisition planes and image mosaic construction. (A) The pCLE miniprobe tip is placed perpendicular to the surface of the tissue sample, enabling images to be obtained in an "en-face" plane. (B) A large pCLE image mosaic of colonic crypts was constructed by stitching adjacent video frames to its corresponding spatio-locations to give a representative panoramic view of the tissue architecture.

Figure 2.6 pCLE image mosaic and histology of FS-stained human colonic mucosa (A) pCLE image mosaic of crypts with dark central lumens. The fluorescent honeycomb patterns surrounding each crypt are capillary networks. (B) Histology image of the corresponding crypts.

Figure 2.7 eCLE images and histology of AH-stained human colonic mucosa (A) eCLE image of colonic crypts. The nuclei of individual colonic epithelial cells are depicted by the red arrow. (B) Magnified eCLE image of a colonic crypt with basal nuclei polarity. (C-D) Histology slides of images A and B depicting similar morphological architecture at the same magnification (20x). [Adapted from Sanduleanu et al., (X)]

Figure 2.8 Miami classification for esophageal epithelium on pCLE image acquisition [Adapted from Wallace et al., (XX)]

Figure 2.9 Miami classification for colorectal lesions on pCLE image acquisition [Adapted from Wallace et al., (XX)]

Figure 2.10 Miami classification of the biliary tract from pCLE image acquisition [Adapted from Meining et al. (XX)]

Figure 2.11 The additional of pCLE description of inflammatory stenosis forms the Paris classification, a modification of the Miami classification [Adapted from Meining et al., (XX)]

Figure 2.12 pCLE images of (A) normal bladder urothelium depicting an umbrella cell layer: note the characteristic large polygonal-shaped cells; (B) Low grade urothelial carcinoma with crowding of uniform-appearing cells; (C) Fibrovascular stalk with a thickened endothelial layer; (D) Cross...
sectional view of the fibrovascular stalk with erythrocytes in the vascular core; (E) High-grade urothelial carcinoma with pleomorphic and distorted sheet of cells. (F) Distorted fibrovascular stalk with variation in vascular cores sizes. [Adapted from Wu et al, (X)] …………………………………… 33

**Figure 2.13** nCLE images of normal pancreatic tissue and intraductal papillary mucinous neoplasam. (A) Normal pancreatic tissue depicts thin dark bands with reticular pattern. (B-C) Histology images of tumor tissue cystic dilatation of ducts with papillary proliferation. (D-E) nCLE images of the tumor with papillary projections. [Adapted from Konda et al, (X)] ……………………………..……. 34

**Figure 2.14** pCLE images of disrupted elastic fibres (yellow circles) (A-C) with patients with lung emphysema. Image D depicts the “snapping” of elastic fibres on consecutive still pCLE images. On the background, fluorescent blobs of macrophages were visualised which is consistent with chronic tobacco smoking. [Adapted from Newton et al, (X)]………………………………….……. 36

**Figure 2.15** (A & B) A laparoscopic view of transvaginally inserted flexible robot i-Snake® performing retroflexion in the pelvis. (C) Tip of the pCLE probe deployed against the liver, (D) spleen and (E) peritoneum (F) pCLE image of liver hepatocytes (G) pCLE image of splenic cords of Billroth; (H) Parietal peritoneum of the abdominal wall with the arrow depicting a blood vessel. [Adapted from Newton et al,(X)] ……………………………………………………………… 43

**Figure 2.16** System set-up of rigid CLE probe and AH-stained human brain tissue CLE morphology (A) The rigid CLE has a shaft diameter of 7mm. (B) Human brain tissues containing normal and adjacent tumor section. (C-D) Normal brain tissue with with neuron or microglial cells with appendices (arrows). (E-F) Tumor tissues reveals excessive growth with atypic nuclei and abnormal nuclear-to-cytoplasm ratio. [Adapted from Foersch et al., (X)] ……………………………………………………………… 44

**Figure 2.17** Venn diagram depicting the envisaged intraoperative pCLE application and subsequent decision-making processes. ……………………………………………………………………………… 46

**Figure 2.18** pCLE image depicting blood vessels (red arrows) with intraluminal erythrocytes (dark particles) on (A) Barrett’s segment; (B) Normal bladder mucosa; (C) Normal bowel mucosa. [Image
A adapted from Konda et al.; Image B provided by Dr. Joseph Liao, Stanford University, CA, USA.

**Figure 2.19** Intraoperative images of anatomical neck dissection in a porcine model. (A) Vertical neck incision was performed; (B) Self retractors were placed and a suture sling was used to retract the strap muscle. (C) Thyroid gland with a pCLE Mini-O miniprobe deployed perpendicular to its surface for image acquisition. Additional pCLE imaging was performed on the thymus (black arrow), trachea (yellow arrow), strap muscles (arrowhead) and (D) on the internal jugular vein (black arrow) and carotid artery (yellow arrow).

**Figure 2.20** Real-time pCLE image mosaics of soft tissue morphology were readily discernible. (A) Polygonal shaped fat cells; (B) Cylindrical shaped longitudinal muscle cells of the esophagus; (C) striated cells of strap muscles; (D) Follicular cells of the thyroid gland using topical application of 0.05% AH as an additional contrast agent; (E) Fluorescent-stained thyroid follicles; (F) Opaque appearance of colloid in the central lumen of thyroid follicle; (G) Thymus lobule with a surrounding vascular septa.

**Figure 2.21** pCLE equipment and sterile transparent drape set-up. (A) The entire miniprobe is sheathed with the drape. The distal end of the drape is folded backwards and the small opening is obliterated with the blue tape. (B) The blue tape is secured around the fibrebundle so that interface between probe tip and sheath is taut. (C) pCLE image acquisition is performed utilising 2.6-mm diameter Ultra-Mini-O™ miniprobe. (D) pCLE image mosaic of parathyroid cells with the sterile drape. (E) pCLE image mosaic without the drape.

**Figure 2.22** Screenshots of a video tutorial designed to train inexperienced surgeons on Miami classification of neoplastic and non-neoplastic colorectal lesions.

**Figure 3.1** Experimental workflow. (A) Freshly excised human mastectomy specimen. (B) Specimen carefully ‘breadsliced’ at 3-5 millimeter intervals. (C) Tissue samples obtained from the section containing macroscopically visible disease (red box) and from adjacent normal tissues (blue box) at...
least 30 mm away from the diseased site. Specimens were immersed separately in AH 0.01% solutions for 60 seconds. ................................................................. 61

**Figure 3.2** Image acquisition of tissue cut-outs. (A) pCLE system; (B) Handheld pCLE miniprobe. The 488-nm excitation laser transmitted onto the tissue surface through a bundle of more than 10 000 optical fibers. Tissue samples imaged *en face* (insert). (C) An example of pCLE image mosaic created in the direction of probe movement. ................................................................. 62

**Figure 3.3** pCLE images of AH-stained breast tissues at (A) 0.01%; (B) 0.05%; and (C) 0.08% concentrations. ................................................................. 65

**Figure 3.3** Each image depicts the morphological appearance on pCLE image (left panel) and the histology image of the corresponding sample (right panel). (A) A well-defined breast lobule with an aggregate of acini. Each acinar depicts a typical target-like appearance with dark-colored central lumen surrounded by a thin layer of homogenously lined fluorescent dots (nuclei of epithelium). (B) Fibrous connective tissue consisting of numerous fine, grey and linear strands of collagen fibers on a relatively acellular background. (C) Fibrous connective tissue with a mildly cellular stroma. Multiple small and discreet bright dots are seen scattered through the stroma. These represent the nuclei of individual fibroblasts. (D) Three adjacent dilated benign breast ducts are seen as larger-sized luminal structures with a thin layer of fluorescent dots. The lumen remained dark-coloured as it is acellular. (E) Elastic fibers visualized as wavy, bright-colored strands which can give an impression of a haphazard architecture. However, the background tissue remains scantily cellular as depicted by the sparsely populated nuclei. (F) Adipose tissue with numerous fat cells depicted as dark-colored, polygonal-shaped cells with thin well-defined bright borders. The nuclei of individual fat cells are visualized as distinct dots at its borders. ................................................................. 66

**Figure 3.4** Each image comprise of a pCLE image (left panel) and a histology image (right panel) of the corresponding sample. (A) Ductal carcinoma in situ: The epithelial lining of this duct appears markedly thickened and the luminal border of the epithelium is seen to be encroaching into the lumen. The entire duct appears markedly thickened and distended. (B) Invasive ductal carcinoma: Multiple
irregularly organized clusters of fluorescent dots are seen infiltrating the stroma giving rise to a haphazard and disorganized appearance. Instead of the small and discreet bright dots of fibroblast nuclei, these clusters are larger in size and contain multiple fluorescent dots. A dense fibrous tissue response known as “desmoplastic reaction” is seen adjacent to these infiltrates producing a scirrhous-like appearance. (C) Invasive ductal carcinoma: Broad sheet of densely packed tumour cells. (D) Ductal carcinoma in situ: The lumen of the duct is occupied by an island filled with multiple fluorescent dots. (E) Invasive ductal carcinoma: another example of multiple clusters of tumour cells with stromal infiltration. (F) Invasive lobular carcinoma: Tumour cells are observed to be loosely dispersed in single linear rows. These are known as Indian file pattern, a pathognomic sign of classical type invasive lobular carcinoma. (G) Metaplastic carcinoma with spindle-cell morphology: A relatively rare variant of invasive carcinoma with tumour cells seen here as adopting a spindle/comma shaped appearance. (H) Invasive tubular carcinoma: Tumour cells producing a ‘target cell’ appearance in the midst of a markedly hypercellular and elastotic background.

Figure 3.5 New pCLE classification for neoplastic and non-neoplastic breast morphology with adjoining magnified views of image mosaics for visual comparison.

Figure 4.1 Freshly excised fluorescein-stained wide local excision (WLE) breast specimen with the surrounding margins inked. (A) Tumor site (shown between the forceps) identified during vertical sectioning. (B) Small cut-out containing the tumor for pCLE imaging. (C) Small WLE specimen with tumor approximating the margins. The tumor was imaged in situ as cut-outs might interfere with subsequent margin assessment.

Figure 4.2 Length of image mosaic was calculated along the centre-point in the longitudinal axis as depicted by the yellow dotted lines.

Figure 4.3 pCLE images and corresponding histology of invasive ductal carcinoma. (A) pCLE mosaic acquired from FS-stained tumor sites demonstrated a markedly hyperfluorescent appearance. These findings were in line with the presence of dense fibrous tissue response on the corresponding site on histology slides. Within these hyperfluorescent regions, multiple haphazardly arranged shades of white
opaque patches were discernible giving rise to a heterogeneous texture. Additionally, numerous stellate-shaped dark spaces were seen interspersing this region and are likely to represent the ground substance of the stroma. (B) Invasive ductal carcinoma on histology with desmoplastic reaction visible in the stroma. (C) pCLE mosaics following additional staining with topical AH, the characteristic clusters and streaks of infiltrating tumor cells were seen embedded on these heterogeneous shades with the adjacent dark stellate-shaped spaces preserved.

Figure 4.4 pCLE images of infiltrating tumor cells from an invasive ductal carcinoma specimen. (A) pCLE mosaic of heterogeneously fluorescent regions depicting areas of tumor-induced fibrosis; (B) Histology image of infiltrating tumor cells evoking a desmoplastic reaction in the stroma; (C) pCLE mosaics of the corresponding area with bands and clusters of tumor cells (yellow arrows) surrounded by tumor-induced opaque white fibrosis.

Figure 4.5 pCLE images of infiltrating tumor cells admixed with adipocytes. (A) FS-stained specimens depicting a heterogeneously appearing opaque stroma (yellow arrows) suggesting the presence of pathological fibrosis around aggregates of dark-colored adipocytes. (B) Histology of the corresponding site containing invasive ductal carcinoma. (C) Following topical staining with AH, clusters of tumor cells (yellow arrows) were seen embedded on the opaque stroma.

Figure 4.6 pCLE images of ductal carcinoma in situ (DCIS) (A-B) Histology images of low grade DCIS. (C) The distended ducts of DCIS are clearly evident on FS-stained tissues. There are opaque material within the lumen of these ducts which might represent the presence of comedonecrosis. (D) Hypercellular epithelium encroaching the lumen (yellow arrows) were discernible. The presence of nuclei on the edges of these dark oval shaped structures confirms the presence of glandular structures and not that of adipocytes.

Figure 4.7 pCLE images of the stromal component of normal breast tissues. (A-E) Following topical AH staining of FS-stained tissues, the borders of adipocytes are well delineated with nuclei visible on its edges. The fibrous tissues here appear relatively homogenously stained (yellow arrows) as
compared to that of tumor-induced fibrosis. The haphazard and whirl-like background seen on tumor stroma is absent. (F) pCLE mosaic of adipocytes on FS-stained tissues prior to AH application. ….. 97

**Figure 4.8** The proposed algorithm for intravenous FS during preparation for pCLE cavity scanning. ........................................................................................................................................................................ 102

**Figure 5.1** (A) pCLE system set-up with an example of an image mosaic depicted on the display screen. (B) pCLE miniprobe emitting a 488-nm, blue light excitation wavelength laser at its distal tip. (C) The tip of the pCLE miniprobe is placed perpendicular to the cut-surface of a freshly excised parathyroid adenoma specimen. ........................................................................................................................................................................ 108

**Figure 5.2** pCLE image mosaics of parathyroid morphology stained at various AH concentrations. (A) Excessive fluorescence noted at 0.05% concentration; (B) Individual nuclei delineated when 0.01% was used; (C) Some nuclei remain unstained at 0.005% and construction of image mosaics were not possible. ........................................................................................................................................................................ 111

**Figure 5.3** Comparison of morphological architecture of hypercellular parathyroid tissues seen on histology (left panel) and pCLE image (right panel) of the corresponding sample. (A & B) Hypercellular parathyroid parenchyma are seen here as numerous fluorescent dots organized in little nests and appear more densely packed in Image B. Fat cells are invariably absent in parathyroid adenoma and sparsely admixed within the parenchyma in parathyroid hyperplasia samples. (C & D) Microfollicular variant of parathyroid hyperplasia. (E & F) The parathyroid vascular network, represented by numerous fibrovascular septa are characterized here by interconnecting dark-coloured, hollow-looking, relatively acellular cord-like / slit-like spaces (yellow arrows) that traverse the densely packed parenchymal cells giving rise to a trabeculated appearance. ........................................................................................................................................................................ 112

**Figure 5.4** Comparison of cystic changes on hypercellular parathyroid tissues seen on histology (A) and pCLE images (B and C). Histology from a section on a large 20-mm parathyroid adenoma depicting multiple small cystic spaces interspersed with parenchymal cells. These are represented on pCLE images by multiple pockets of capacious, amorphous and acellular spaces. ......................... 114
**Figure 5.5** Magnified views of pCLE mosaics of parathyroid microfollicular architecture interspersed with adipocytes. ................................................................. 115

**Figure 5.6.** Magnified views of pCLE mosaics of fibrovascular spaces within the parathyroid parenchyma. Nests of parenchymal cells seen between these spaces. ............................................. 116

**Figure 5.7** Comparison of non-parathyroid tissues on histology (left panel) and pCLE images (right panel). (A) Thyroid follicles are seen as dark-coloured, luminal structures, lined by a discreet layer of fluorescent dots depicting that of an epithelium. Several lumens are seen permeated with opaque grey-like substance which connotes the presence of colloid material. (B) A thyroid follicle with adjacent fibrous stroma. (C) Adipose tissues populated by dark-colored, polygonal-shaped cells. (D) Fibrous tissues that populates the thyroid stroma. ................................................................. 117

**Figure 5.8** pCLE images of thyroid tissues depicting follicles of various sizes and shapes consistent with that of a multinodular goitre specimen. ................................................................. 119

**Figure 5.9** Examples of errors encountered during pCLE image interpretation using typical thyroid follicles for comparison (A). B, Thyroid follicles mistakenly identified as adipocytes due to the visibly sparse epithelial nuclei. C, Parathyroid microfollicles misinterpreted as that of the thyroid. D, Adipocytes with a central cluster of fibroblast nuclei misidentified as parathyroid tissues. E, Cystic spaces of parathyroid tissues thought to represent thyroid follicles. F, Multinodular goitre with follicles of varying sizes misinterpreted as parathyroid tissues due to adjacent degenerative changes................................................................. 121

**Figure 5.10** Histogram of difficulty levels of assessment on pCLE image interpretation measured on a five-point Likert scale grouped according to (A) Tissue type; (B) Pathologists; (C) Surgeons. Data are presented as mean ± SD. *ANOVA comparisons of all three groups were significant at p<0.05. There was significant pair-wise differences between the mean scores and all tissue types for pathologists (p<0.05); and between thyroid and fibrofatty tissues for surgeons (p<0.05). ............................................. 124
**Figure 6.1** pCLE system set-up in the operating theatre. (A) The display screen is placed within the vicinity of the operating surgeon. (B) The pCLE miniprobe is draped with a sterile transparent sheath that is reinforced with a layer of Tegaderm at its distal tip to ensure uniform abutment of the miniprobe tip with the PG surface during image acquisition. (C-D) In patients undergoing parathyroidectomy, further imaging is performed following excision or devascularisation of the respective PG.

**Figure 6.2** Flow diagram of structured evaluation of vascular morphology for each pCLE video clip.

**Figure 6.3** Classification to facilitate grading of pCLE video stability taking into account aspects related to video movement and visual strain.

**Figure 6.4** pCLE images of PG vascular morphology with the vascular pedicle preserved. The respective vasculatures are denoted by the yellow arrows (A) Interconnecting network of capillaries; (B) Large vessel with individual erythrocytes (red arrow); (C) Single vessel in between aggregates of intraparenchymal fat cells. (D) Branching and tortuous looking vessels; (E) Overlapping vessels; (H) Branching vessels with adjacent adipocytes. Asterisk (*) denotes fat cells.

**Figure 6.5** pCLE images of PG vascular morphology following devascularisation or excision. (A) Network of capillaries with empty and contracted lumens (dark coloured); (B) Empty branching vessels interspersing the parenchymal adipocytes; (C) Parallel empty vessels; (D) FS containing network of capillaries with no erythrocytes visible within the lumen; (E) Single vessel with FS within. No erythrocytes seen; (F) Single vessel containing FS with static erythrocytes visible (red arrow).

**Figure 6.6** Magnified views of pCLE image mosaics of blood vessels and individual erythrocytes.

**Figure 6.7** An example of pCLE image mosaic of the vast networks of capillaries on preserved PG.
Figure 6.8 pCLE image mosaic depicting PG branching vessels and fibrous connective tissue (bottom half). ………………………………………………………………………………………………………………………… 146

Figure 6.9 pCLE images depicting presence and absence of vascular flow on PG from three patients (A & B) Empty vessels seen on both PGs from Patient 1; (C) Single vessel with vascular flow from Patient 4; (D) Parallel empty vessels on the same PG from Patient 4; (E) Large and small vessels with vessel stasis from Patient 7; (F) Network of capillaries with sluggish flow on the same PG from Patient 7. ………………………………………………………………………………………………………………………… 150

Figure 7.1 This flow-diagram depicts the three phases of research development of pCLE image towards real-time applications in surgery. This thesis established the foundations whereby promising intraoperative applications were discovered as shown in Phase 1 (highlighted in yellow). Additionally, it laid the building stones for Phase 2 which comprises of systems evaluation using technological adjuncts to augment image acquisition. This constitutes our next research priority prior to embarking on the relevant clinical trials………………………………………………………………………… 160

Figure 7.2 A selection of endomicroscopy probes to be tested for optical tradeoffs in the aforementioned surgical applications. (a) A bare-tipped 10k core, 0.6 mm diameter Sumitomo bundle; (b) A 30k core, 0.5 mm diameter Fujikura bundle with an integrated x2 magnification distal lens assembly (1.4 mm in diameter); (c) an ultra-flexible 17,000 core Schott leached imaging bundle with a diameter of 1.2 mm; (d) a semi-rigid, 100k core Fujikura fibre bundle, 1.7 mm in diameter; and (e) a 50k core Schott rigid image conduit, 3.2 mm in diameter. [Image courtesy of Dr Michael Hughes, Research Associate, The Hamlyn Centre, Imperial College London] ……………………………………… 162

Figure 7.3 (a) Robotic manipulator system set-up comprising of a light weight KUKA© robot with the force adaptive control instrument mounted at the end-effector of the robotic arm with CLE image mosaic construction obtained from a simulated breast cavity; (b) During CLE image acquisition, the position of robot in 3-dimensional space were recorded and surface reconstruction with mapping of the CLE image mosaic were mapped to its corresponding location in the simulated breast cavity. [Adapted from Simaiaki et al, (X)] …………………………………………………………………………………………………………………………………………………………… 163
List of tables:

Table 2.1 A summary of the optical specifications and trade-offs between probe sizes and resolution of some of commonly used pre-clinical and clinical pCLE miniprobes that are commercially available. Sourced from www.cellvizio.net ................................................................. 18

Table 2.2 Original studies assessing the diagnostic accuracy of CLE on differentiation between neoplastic from non-nonplastic colorectal lesions. ................................................................. 28

Table 2.3 Original articles assessing the diagnostic accuracy of pCLE on differentiation between benign and malignant biliary strictures. ................................................................. 30

Table 3.1 Correlation between pCLE image interpretation and gold standard histology............. 76

Table 3.2 Accuracy in pCLE images 1 to 10, 11 to 20, 21 to 30, 31 to 40, and 41 to 50 for each observer and overall. .................................................................................................................. 77

Table 4.1 Baseline characteristics of patients undergoing BCS, intravenous FS dosage and circulation time. .................................................................................................................. 92

Table 5.1 Correlation between tissue type identification on pCLE images and histology. .......... 120

Table 5.2 Correlation between pCLE image interpretation and histology on parathyroid and non-parathyroid tissues. ......................................................................................................... 122

Table 5.3 Comparison of mean level of difficulty of assessments on pCLE image interpretation measured on a five-point Likert scale grouped according to tissue type and specialty. Data are presented as mean ± SD. * denotes p value of <0.05. .................................................................................. 123

Table 6.1 Interobserver agreements on components of vascularity assessment, and correlation between pCLE video stability and level of confidence. ................................................................. 147
Table 6.2 Correlation between pCLE vessel flow assessments and intraoperative evaluation on viability status of parathyroid glands from Cohort 1. …………………………………………………………… 148

Table 6.3 Post-operative corrected calcium and parathyroid hormone levels compared to vessel flow and flow rate assessments on pCLE videos from Cohort 2. ………………………………………… 149
List of acronyms:

2D Two-dimensional
3D Three-dimensional
4D-CT Four-dimensional computed tomography
AH Acriflavine hydrochloride
ANOVA Analysis of variance
BCS Breast conserving surgery
BE Barrett’s esophagus
BJS British Journal of Surgery
CBD Common bile duct
CE Chromoendoscopy
CLE Confocal laser endomicroscopy
CLSM Confocal laser scanning microscopy
CND Central neck dissection
CT Computed tomography
DCIS Ductal carcinoma in situ
eCLE Endoscopic confocal laser endomicroscopy
ERCP Endoscopic retrograde cholangiopancreatography
EUS Endoscopic ultrasound
FC Fat cells
FDA Food and drug administration (USA)
FNA Fine needle aspiration
FOV Field of View
FPS Frames per second
FS Fluorescein sodium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumour</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haemotoxylin and Eosin</td>
</tr>
<tr>
<td>HD</td>
<td>High definition</td>
</tr>
<tr>
<td>HRCT</td>
<td>High resolution computed tomography</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive ductal carcinoma</td>
</tr>
<tr>
<td>ILC</td>
<td>Invasive lobular carcinoma</td>
</tr>
<tr>
<td>ioPTH</td>
<td>Intraoperative parathyroid hormone assay</td>
</tr>
<tr>
<td>KS</td>
<td>Mr Kumuthan Sriskandarajah, Clinical Research Fellow</td>
</tr>
<tr>
<td>LSU</td>
<td>Laser scanning unit</td>
</tr>
<tr>
<td>MGD</td>
<td>Multiglandular disease</td>
</tr>
<tr>
<td>MRCP</td>
<td>Magnetic resonance cholangiopancreaticography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NBI</td>
<td>Narrow band imaging</td>
</tr>
<tr>
<td>nCLE</td>
<td>Needle based confocal laser endomicroscopy</td>
</tr>
<tr>
<td>NIHR</td>
<td>National Institute of Healthcare Research</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>pCLE</td>
<td>Probe-based confocal laser endomicroscopy</td>
</tr>
<tr>
<td>PG</td>
<td>Parathyroid glands</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>REC</td>
<td>Research ethics committee</td>
</tr>
<tr>
<td>RFS</td>
<td>Radiofrequency spectroscopy</td>
</tr>
<tr>
<td>ROLL</td>
<td>Radioguided occult lesion localization</td>
</tr>
<tr>
<td>SARS</td>
<td>Society of Academic and Research Surgery</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
<tr>
<td>SS</td>
<td>Sestamibi scintigraphy</td>
</tr>
<tr>
<td>TEMS</td>
<td>Transanal endoscopic microsurgery</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TPC</td>
<td>Mr Tou-Pin Chang, Clinical Research Fellow</td>
</tr>
<tr>
<td>TURBT</td>
<td>Transurethral resection of bladder tumour</td>
</tr>
<tr>
<td>USS</td>
<td>Ultrasound scan</td>
</tr>
<tr>
<td>WLC</td>
<td>Wide light cystoscopy</td>
</tr>
<tr>
<td>WLE</td>
<td>Wide local excision</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction and Overview

1.1 Introduction

Recent improvements in fibre optic technology and miniaturization of optics have enabled the development of a probe-based confocal endomicroscope (pCLE) that is capable of producing high resolution in situ in vivo imaging of cellular morphology by optical sectioning. These probes are designed to be inserted into one of the working channels of the endoscope, enabling tissues to be imaged in situ without the need for biopsy. This method of obtaining real-time ‘virtual histology’ images has the potential to facilitate clinicians make immediate decisions pertaining to the immediate management of their patients. Since the inception of this technology, scientific and clinical communities worldwide are keen for pCLE to fulfil its full potential as evidenced from the vast number of publications pertaining to envisaged applications in the gastrointestinal tract, biliary tree, respiratory tract, urology and more recently, on pancreatic cysts.

There is however an obvious gap in the evaluation of pCLE as a potential imaging tool for surgical applications. One of many challenges a surgeon faces in the operating theatre is the inability to recognise inconspicuous disease on the tissues being operated on. When this pertains to breast cancer surgery, tumor cells could potentially be left behind resulting in tumor recurrence, poorer prognosis or reoperations. During parathyroid surgery to remove the diseased parathyroid glands, distinguishing these glands from adjacent tissues can be difficult to distinguish thereby frozen sections are occasionally performed to obtain real-time diagnostic information pertaining to the tissue entity being queried. These are time-consuming, costly and might not be reliable. Preservation of viable parathyroid glands is essential to ensure adequate parathyroid function post-operatively. However, devascularisation can occur as a result of inadvertent trauma and this might not be apparent at the time of surgery.
The aim of this thesis is to systematically explore and evaluate the ability of pCLE to image morphological architecture of tissues pertinent to the aforementioned intraoperative applications and assess the feasibility of surgeons to interpret these images. The strengths and limitations of pCLE imaging are identified objectively thereby forms the scientific basis for future work to be carried out to fulfil its full potential as an imaging adjunct that facilitates decision-making during surgery.

1.2 Thesis chapter synopsis

Chapter 1: This provides an overview on the rationale of technological assessment on intraoperative applications where significant gaps currently exist, the key scientific contributions of this thesis and a list of academic achievements generated from this thesis.

Chapter 2: This includes a comprehensive overview of pCLE, describing its development, potential clinical applications and challenges associated with deployment of pCLE miniprobe. The chapter also discusses the current gap that exists pertaining to the use of pCLE for surgical applications. Real-time morphological information pertaining to tumor margin status, tissue type identity and tissue viability status has been identified as the three “core surgical themes” that warrant exploration. An overview of the current intraoperative challenges relevant to these themes such as assessment of tumor-free cavity margin status in breast cancer surgery; identification of parathyroid glands during bilateral neck dissection; and preservation of viable parathyroid glands during total thyroidectomy is presented alongside the envisaged clinical impact pCLE has on intraoperative decision-making. Lastly, the results of two pilot studies are summarised – the presence of dried blood on the tissue surface and the use of a sterile transparent sheath did not interfere with pCLE image acquisitions. Surgeons were able to interpret pCLE images obtained from the lower gastrointestinal tract with high accuracy rates (>90%). Whilst these studies support the notion of an in situ in vivo pCLE image acquisition and interpretation by the surgeon, its ability to image tissue morphological architecture relevant to the aforementioned applications warrants systematic evaluation and thereby is the focus of the following chapters in this thesis (Figure 1.1).
Chapter 3: This chapter describes a prospective, cross-sectional, observational study that was carried out on freshly excised human breast specimens containing 50 tumor and 21 non-tumor sections. Utilizing acriflavine hydrochloride as the fluorescent agent and histology slides of the corresponding sections for gold standard comparison, morphological features of invasive and non-invasive breast cancers were readily visualized and distinguished on pCLE images. Following evaluations with two experienced breast pathologists, a novel classification that consists of pCLE descriptions of glandular and stromal morphology components was developed. Seventeen surgeons and pathologists were invited to complete a pattern recognition training session based on this classification. Following completion of their training, blinded assessments on their accuracies of image interpretation was carried out on 50 anonymous pCLE images (25 neoplastic and 25 non-neoplastic).

The mean accuracies for surgeons and pathologists were 94% (range 90-100%) and 92% (range 84-98%), respectively. The mean interobserver agreement for pathologists were ‘excellent’, κ=0.82 (95%CI, 0.79-0.85); and ‘substantial’ for surgeons, κ=0.74 (95%CI, 0.70-0.78). Given the flexibility and size of pCLE miniprobe, there might be potential role for pCLE to be deployed against the cavity walls created during breast conserving surgery for breast cancer. Evidently, real-time identification of residual tumor cells on these walls could potentially be used to guide decision-making pertaining to further excision during the index surgery.

Chapter 4: This chapter evaluates the use of intravenous fluorescein independently or in conjunction with topical acriflavine hydrochloride to highlight morphological architecture of neoplastic and non-neoplastic human breast tissues. Ten patients undergoing breast conserving surgery for breast cancer were given intravenous fluorescein intraoperatively. pCLE image acquisition was performed on tumor and non-tumor specimens post-operatively before and after further staining with topical acriflavine.

Tumor stromal morphology was depicted as markedly heterogeneous fluorescent regions with relatively haphazard appearance as compared to that the homogenous appearance of non-tumor sections. Further staining with topical acriflavine showed that the stroma adjacent to infiltrating bands
of neoplastic cells project an opaque grey-like appearance akin to that of a halo effect. These findings were consistent with the pathognomonic findings of stromal desmoplastic reaction in invasive breast cancer. The mean length of mosaics created from the combined fluorescein- and acriflavine-stained sections was significantly longer than that of acriflavine-stained only sections. Evidently, the use of fluorescein sodium increases the overall morphological architecture visualised; allows uniform staining of all tissue layers; and consequently, increases the length of pCLE mosaics created. The addition of intravenous fluorescein as an additional fluorescent agent could potentially facilitate acquisition of higher quality mosaics thereby increases the yield of residual disease detection during cavity wall scanning, thus warrants intraoperative in situ evaluation.

Chapter 5: This chapter details another exciting prospective, cross-sectional, observational study on the role of acriflavine hydrochloride as a potential fluorescent agent to facilitate tissue differentiation during parathyroid surgery. Freshly excised parathyroid and non-parathyroid (thyroid and fibrofatty) tissues were obtained from 35 patients that underwent parathyroidectomy and thyroidectomy for benign and malignancy diseases. Consistent with histopathology findings, nest-like arrangement of parenchymal cells, fibrovascular septum, microfollicles and cystic spaces of parathyroid tissues were readily identifiable on pCLE images. Similarly, colloid-filled thyroid follicles were easily distinguishable from the polygonal-shaped hyperfluorescent borders of fat cells. Mean accuracy of pCLE image interpretation for pathologists and surgeons were 94% (range 93-94%) and 93% (91-94%), respectively. The mean inter-observer agreement for tissue type identification among pathologists was 'excellent', $\kappa=0.86$ (95%CI, 0.82-0.90); and ‘substantial’ among surgeons, $\kappa=0.80$ (95%CI, 0.75-0.85). In light of these novel findings, intraoperative in situ evaluation of CE’s role in tissue differentiation is warranted.

Chapter 6: This chapter describes the first human clinical trial investigating the feasibility of in situ in vivo imaging of parathyroid gland vascular morphology using pCLE. Twenty patients undergoing parathyroidectomy for primary hyperparathyroidism and total thyroidectomy for benign and malignant disease received 1.5-2.5ml of 10% intravenous fluorescein intraoperatively. For both groups of
patients, pCLE imaging was performed by deploying a sterile-draped pCLE miniprobe onto parathyroid glands with carefully preserved vascular pedicle to ensure viability. However for the parathyroidectomy patients, pCLE imaging was repeated following excision/pedicle obliteration for comparison of viability features on its vasculature.

A wide array of morphological vasculature was readily visualised including single, branching, overlapping vessels of various sizes including capillary networks. Vascular flow on viable glands was depicted by unidirectional, high velocity thrusts of dark-coloured erythrocytes within hyperfluorescent vessels whereas these were absent on non-viable glands. The overall sensitivity, specificity, and accuracy of non-viability on pCLE images was 92% (range 78-100%), 97% (range 90-100%) and 95% (range 85-100%), respectively. Further validation in the total thyroidectomy group showed that preserved parathyroid glands with empty blood vessels visualised demonstrated permanent loss of parathyroid function post-operatively. These findings suggest that visualization of empty or static vessels, especially larger sized vessels, could potentially indicate impending ischaemia on that gland.

Figure 1.1 The experimental chapters develop from three ‘core surgical themes’ highlighted in Chapter 2.
1.3 *The main scientific contributions in this thesis*

The 10 main scientific contributions in this thesis that advance pCLE knowledge, understanding and its potential clinical impact are:

1. The creation of an extensive library of pCLE images depicting neoplastic and non-neoplastic breast morphology; parathyroid and non-parathyroid morphology; and parathyroid vascular morphology.

2. The confirmation that pCLE image acquisition of tissue morphology is feasible when a sterile transparent drape is used as an interface between probe-tip and blood-stained tissue surface.

3. The first description of neoplastic and non-neoplastic changes on glandular and stromal components of the breast on pCLE images; and the resulting scientific interests towards intraoperative applications

4. The first description of parathyroid and thyroid parenchymal morphology on pCLE images; and the resulting scientific interests towards intraoperative deployment.

5. The development and validation of a novel classification system to facilitate recognition of neoplastic and non-neoplastic breast morphology on pCLE images

6. Pattern recognition skills required for pCLE image interpretation can be learnt easily and quickly by surgeons following a structured training session on the basics of histology and corresponding pCLE images.

7. The use of intravenous fluorescein during surgery for pCLE image acquisition is feasible and safe in humans and provides interpretable images of vascular and non-vascular morphology.

8. The first human description of parathyroid gland vascular morphology and morphological features associated with non-viability
9. The findings that patients who develop permanent hypoparathyroidism had empty or static vessels visualised on preserved parathyroid glands during total thyroidectomy

10. The conclusion that further modifications to the pCLE miniprobe’s field of view, resolution and technological adjuncts to improve stability of miniprobe deployment is warranted to facilitate translation into intraoperative use.

1.4 Prizes and publications associated with this thesis

The prizes, awards and grants listed below were awarded to research work included in this thesis:

- **National Institute for Health Research (NIHR) Imperial Biomedical Research Centre (BRC) Funding Award**
  “The Role of Confocal Endomicroscopic Imaging of Fluorescein-Stained Breast Cavity Walls in Detecting Residual Disease Foci following Breast Conserving Surgery”: #P51438 £33,000
  Co-Investigator

- **Society of Academic & Research Surgery (SARS) Bursary 2014**
  Travelling bursary awarded for attendance at Society of Academic and Research Surgery (SARS) meeting, Robinson College, Cambridge University, January 2014

- **Winner, Division of Surgery Research Afternoon Prize, Imperial College London**
  Best plenary presentation at the Annual Divisional Research Afternoon, Imperial College London, December 2013
Chapter 2


Chapter 3


**Chapter 4**

Chapter 5


Chapter 6


Other relevant peer reviewed publications, presentations and conference proceedings
(content not included in this thesis)


Chapter 2: Overview of probe-based confocal laser endomicroscopy

2.1 Evolution of probe-based confocal laser endomicroscopy

2.1.1 Principles of confocal microscopy

The confocal microscope was first invented by Marvin Minsky in 1955 when he was a junior research fellow at Harvard University, and its principles were subsequently patented in 1957 (1). The motivation for its development was to overcome some of the limitations encountered with conventional white light microscopy. The latter utilises a light source to illuminate the tissues evenly and thereby causes the optical pathways of the entire tissue excited at the same time creating an unfocused and blurred image in the unsectioned tissue. In order to eliminate the unfocused background, these specimens inevitably need to be excised, fixed and sectioned to generate 3-10µm thin slices. By doing so, the reflections obtained from tissue illumination are limited to those contained in these thin slices thereby enabling focused and sharp images to be acquired (Figure 2.1).

Evidently, conventional white light microscopy warrants tissue fixation and sectioning, a lengthy process that often requires several days. Recognising these limitations, Marvin described the application of point illumination and a pinhole in front of the optical detector to eliminate out-of-focus signals from being captured. In this case, both the condenser and objective lens had the same focal point hence the term ‘confocal’. The point illumination source is focused directly into the tissue, illuminating a single point (focal plane) at any given time. The resulting illuminations from that focal plane are then passed through the pinhole, which acts as a filter to reject non-focal illuminations from the layers above and below the focal plane (Figure 2.1). Given that only one point in the tissue sample is illuminated at a time, additional lateral and axial scanning is required to create a 2D or 3D image of the tissue specimen. All in all, confocal microscopy allows image acquisition of tissue specimens to be
carried out in real-time with the tissue integrity preserved. The depth and the thickness of the focal plane are determined by the wavelength of the light, the numerical aperture of the objective lens and the optical properties of the specimen.

Figure 2.1 Optical pathways of tissues on conventional white light microscopy and confocal microscopy. (A) The clarity of the image produced by white light microscopy emanated from the pathways created from a thinly sliced sample as no other ‘out of focus’ pathways were created. (B) Confocal microscope illuminates a thick block of sample but only allow pathways created from that optical slice (confocal slice) to be filtered through the pinhole, hence eliminating all other out of focused pathways.
Over the years, confocal microscopy has emerged as a popular imaging tool for high resolution imaging of cells and tissues. However, due to the size of the microscope, most descriptions on in vivo studies were pertinent to clinical applications on accessible tissues and organs such as the eye (2), skin (3-5) and the oral cavity (5-7). There were promising studies pertaining to potential applications in surgery, however these preliminary findings were confined to feasibility work on excised tissues (8, 9) (Figure 2.2). The large microscope objective lens renders application of this technology to in vivo imaging of tissues impossible (10). It is evident that two considerable changes to its optical and physical characteristics are warranted. Firstly, the scanning mechanism and image acquisition rate needs to be faster in order to minimise movement artefacts as tissues within the body are highly susceptible to physiological movements and deformation (10). Secondly, significant miniaturisation on one of its image acquisition component is required to facilitate access to deeper organs where the clinical benefits of its real-time in situ image acquisition could be fully evaluated and ascertained (11).

The ground-breaking advancement in the translation of confocal microscopy to in vivo imaging of tissues emanated from the use of fibre optics in place of a traditional confocal pinhole, to preserve the optical sectioning properties of the conventional confocal microscopy system (Figure 2.2) (12-14). Evidently, we have a system that is now comprised of the light source being separated from the detector pinhole with the optical fibre acting as the intermediate component that increases the versatility for imaging ‘difficult to reach’ regions in the body such as applications pertaining to endoscopy, laparoscopy and minimally invasive open surgery (small incisions) (15). These optical fibres serve as versatile tool to deliver and collect illumination from tissues from a predefined focal plane (confocal) and this is made possible from parallel advancement from its scanning mechanisms which can be performed either proximally or distally to the light source. The former’s location of scanning mirrors is distal to the optical fibres at the end of the endoscope and therefore occurs much faster than the latter which are located proximal to these fibres thereby acquiring narrower images. Whilst the benefits of access are clearly evident, the size constraints imposed by concurrently deployed endoscopes and luminal environment meant that trade-offs are inevitable between the field of view, resolution and probe sizes (16, 17).
2.1.2 Confocal laser endomicroscopy

Over the years, it was increasingly recognised that modifications were needed to increase accessibility of the microscope to *in vivo* tissues and faster image scanning and acquisition rate were required to counter movement artefacts. The introduction of a single mode fibre in place of a conventional confocal pinhole has been the cornerstone for clinical translation of confocal microscopy to in vivo clinical imaging on previously inaccessible endoluminal and intra-abdominal regions (14). The use of an optical fibre as the source and detector pinholes allows separation of the imaging arm from the light source and detector, this increases versatility of the confocal microscope for in vivo imaging (13, 18, 19). Subsequently, the work of Martin Harris and Peter Delaney in the late 1980s and 1990s (20, 21) led to the development of the world’s first hand-held confocal microscope by Optiscan (Notting Hill, Australia) for dermatology and subsequently endoscopic applications. Following that, an endoscope-based confocal laser endomicroscopy (eCLE) was developed commercially in collaboration with Pentax (Tokyo, Japan) with a miniaturized Optiscan confocal endomicroscope incorporated into the distal end of a conventional endoscope (EC-3870K, Pentax, Tokyo, Japan) (Figure 1a). It provides high resolution real time in vivo in situ images at cellular resolution and has been used for imaging in
the upper and lower gastrointestinal tract mucosal imaging (8)(9)(10). Another commercially available system called probe-based confocal laser endomicroscopy (pCLE), for example, Cellvizio® (Mauna Kea Technologies, Paris, France) has been increasingly used for endoluminal applications. Cellvizio® is a miniaturized form of confocal microscope that generally consists of a pCLE miniprobe, a laser scanning unit and personal computer for image data processing and display (Figure 2.3). Its platform utilises laser as its light source and the pCLE miniprobe transports a 488-nm-wavelength blue excitation laser light onto the tissue surface and the resulting emissions between 500 and 650 nm from fluorescing tissues are collected at 12 frames per second, hence resembling video quality (Figure 2.4).

Figure 2.3 (A) Endoscope-based confocal endomicroscopy (eCLE) – Endomicroscope integrated at the distal tip of the endoscope (blue arrow). © 2012, Pentax (B) pCLE Cellvizio stack consisting of a personal computer display (top deck), computer processing unit (middle deck) and a laser scanning unit (lower deck). (C) pCLE miniprobe at the point of entry into a working channel of an endoscope. (D) pCLE miniprobe at the distal end of an endoscope with the distal tip of confocal miniprobe abutting the surface of gastrointestinal mucosa during endoscopy (insert). [Adapted from Chang et al, (22)]
The optical slices, depth of imaging and field of view for each frame depends on the type of pCLE miniprobe used and its intended clinical application (Figure 2.4 and Table 2.1). These pCLE miniprobes are highly flexible and are designed to be introduced into the working channels of standard endoscopes. All images were scanned thought a bundle of more than 10 000 optical fibres with a rate of 12 frames per second, hence demonstrating a real-time video on a display screen. This information is then relayed to a personal computer for image data processing and display using a specially designed software package (Cellvizio, Mauna Kea Technologies, Paris, France) where single video frames were reconstructed into a larger linear static image using “mosaicing”, a post-procedure image reconstruction tool based on a hierarchical framework algorithm that is able to recover a globally consistent alignment of the input frames, to compensate for motion-induced distortions and to capture non-rigid deformations. The resulting image mosaics combine all moving images, cancel motion artifacts, and reconstitute panoramas of the tissue samples (Figure 2.5). At present, pCLE has emerged as the most widely used CLE device as it is more versatile, provides the option of acquiring highly magnified images and is not endoscope-constrained.

Figure 2.4 Images depicting the relative sizes of pCLE miniprobes. (A) The Cholangioflex® probe (left) is used for visualization of biliary strictures and the GastroflexUHD® probe (right) is deployed for upper gastrointestinal tract imaging. (B) Mini-O® pCLE miniprobe, a pre-clinical pCLE miniprobe with similar physical characteristics with a detachable plastic holder to facilitate stable apposition against tissue surfaces.
<table>
<thead>
<tr>
<th>pCLE miniprobe types</th>
<th>Gastroflex, Coloflex</th>
<th>Gastroflex UHD, Coloflex UHD</th>
<th>Cholangioflex</th>
<th>Alveoflex</th>
<th>AQ Flex</th>
<th>Mini-O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intended usage</td>
<td>Clinical</td>
<td>Clinical</td>
<td>Clinical</td>
<td>Clinical</td>
<td>Clinical</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td>Target deployment site</td>
<td>Upper and lower GI tract</td>
<td>Upper and lower GI tract</td>
<td>Biliary tree</td>
<td>Lung</td>
<td>Pancreatic cyst</td>
<td>Pre-clinical studies</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>2.5</td>
<td>2.5</td>
<td>1.0</td>
<td>1.4</td>
<td>0.9</td>
<td>2.6</td>
</tr>
<tr>
<td>Probe length (m)</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>0.9</td>
</tr>
<tr>
<td>Depth of imaging (µm)</td>
<td>70-130</td>
<td>55-65</td>
<td>40-70</td>
<td>0-50</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Field of view (µm)</td>
<td>600</td>
<td>240</td>
<td>325</td>
<td>600</td>
<td>320</td>
<td>240</td>
</tr>
<tr>
<td>Lateral resolution (µm)</td>
<td>3.5</td>
<td>1.0</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Frames per second</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 2.1 A summary of the optical specifications and trade-offs between probe sizes and resolution of some of commonly used pre-clinical and clinical pCLE miniprobes that are commercially available. Sourced from www.cellvizio.net.
Figure 2.5 Orientation of pCLE image acquisition planes and image mosaic construction. (A) The pCLE miniprobe tip is placed perpendicular to the surface of the tissue sample, enabling images to be obtained in an "en-face" plane. (B) A large pCLE image mosaic of colonic crypts was constructed by stitching adjacent video frames to its corresponding spatio-locations to give a representative panoramic view of the tissue architecture.
2.1.3 Contrast agents for pCLE imaging

pCLE image acquisition of tissues requires the use of fluorescent contrast agents to enhance visualization of cells. These contrast agents can be administered intravenously or topically. Intravenous fluorescein sodium 10% (FS) is the most widely used contrast agent in pCLE imaging. It is Food and Drug Administration (FDA) approved for diagnostic fluorescein angiography or angioscopy of the retinal vasculature. Although its use in pCLE is considered off-label, its safety record is well reported in numerous pCLE clinical trials (23). Utilizing intravenous FS, morphological structures of tissues are readily visualised within several seconds of administration and the fluorescence lasts for approximately 45 minutes. This however varies depending on the richness of vascular supply of the tissues imaged. Intravenous FS highlights the tissue vasculature and the surrounding extravascular spaces (Figure 2.6). The differences in fluorescence intensity between cellular constituents allow us to identify morphological architectures such as crypts (colon), gastric pits (stomach), gap junctions (small intestines) and network of capillaries (gastrointestinal tract, biliary tree, pancreatic cysts). It does not however highlight the nucleus of individual cells and thereby unable to confer information pertaining to degree of tissues. The intravenous dose required vary between 2.5 to 10ml although most studies report that 2.5 to 5.0ml of intravenous FS given as a bolus injection followed by saline flush would suffice. It provides uniform staining to all layers of tissues and additional top-up doses are rarely needed during the period of endoscopy.

Whilst severe adverse reactions to intravenous fluorescein contrast agent are very rare, many patients report transient (1-2 hours) yellowish discolouration of the skin and mucous membranes, and bright yellow coloured urine for 1-2 days. In the most recent large scale study on intravenous fluorescein for ophthalmology (11898 patients) nausea and vomiting occurred 0.7% and 0.4% respectively. In this study, there were no complications more significant than an urticarial rash (0.2%) (24). A recent smaller safety study of intravenous fluorescein in CE showed no serious complications in 410 consecutive patients (25), and a study of 2272 patients in 16 centres undergoing CE procedures yielded no serious events, with mild events such as nausea and vomiting, injection site erythema, transient hypotension without shock, diffuse rash or mild epigastric pain occurring in 1.4% (23).
Our institution’s previous experience with intravenous FS for pCLE imaging to date has been consistent with the published literature. We had first-hand experience of using 2.5-5ml of intravenous FS to image pCLE changes in mucosal morphology in patients with radiation proctitis at Royal Marsden Hospital (REC No. 11/SC/0036) and in patients with lung parenchymal diseases at Royal Brompton Hospital (09/H0708/18) (26). In both studies, no adverse reactions were reported and all patients demonstrated good tolerance following intravenous administration of FS.

Aside from intravenous FS, topical application of acriflavine hydrochloride (AH) can also be used to stain the tissues. It is compatible with the excitation wavelength laser used and it predominantly stains the nuclei of individual cells (Figure 2.7). The most commonly used dose is 0.05% and it is applied onto the tissue by using a spraying catheter (27, 28). It also demonstrates slight affinity to connective tissues in the stroma and therefore stains the extracellular components of tissues. Utilizing topical AH, morphological architecture of the gastrointestinal tract (27), respiratory epithelium (29) and brain

Figure 2.6 pCLE image mosaic and histology of FS-stained human colonic mucosa (A) pCLE image mosaic of crypts with dark central lumens. The fluorescent honey-comb patterns surrounding each crypt are capillary networks. (B) Histology image of the corresponding colonic crypts.

Aside from intravenous FS, topical application of acriflavine hydrochloride (AH) can also be used to stain the tissues. It is compatible with the excitation wavelength laser used and it predominantly stains the nuclei of individual cells (Figure 2.7). The most commonly used dose is 0.05% and it is applied onto the tissue by using a spraying catheter (27, 28). It also demonstrates slight affinity to connective tissues in the stroma and therefore stains the extracellular components of tissues. Utilizing topical AH, morphological architecture of the gastrointestinal tract (27), respiratory epithelium (29) and brain
tissues (30) could be delineated on CLE images and correlates well with histology. The staining effect is immediate but limited to superficial layers. The unbound fluorescence needs to be flushed away to minimise artefacts. Additional top-up doses are not required and no adverse events have been reported to date. It has been used in conjunction with chromoendoscopy and no interference with the latter’s stains has been reported. Another topical agent, cresyl violet is a cytoplasmic stain used to outline the nucleus. Similar to AH, it is applied onto the tissue surface and immediate staining of morphology is conferred. The depth of staining is limited to the superficial layers.

Figure 2.7 eCLE images and histology of AH-stained human colonic mucosa (A) eCLE image of colonic crypts. The nuclei of individual colonic epithelial cells are depicted by the red arrow. (B) Magnified eCLE image of a colonic crypt with basal nuclei polarity. (C-D) Histology slides of images A and B depicting similar morphological architecture at the same magnification (20x). [Adapted from Sanduleanu et al, (27)]
2.2 Overview of the potential clinical applications for pCLE

2.2.1 Overview

The potential endoluminal clinical applications for pCLE are broad, ranging from those within the gastrointestinal tract; surveillance of Barrett’s esophagus, identification of malignant biliary strictures, detection of neoplastic colorectal polyps; respiratory bronchial tree: assessment of respiratory tract lesions; urological tract: detection of neoplastic bladder lesions; pancreas: evaluation of pancreatic cysts; and respiratory tract: assessment of epithelial lesions. The majority of these were pertinent to differentiation between neoplastic and non-neoplastic tissues during elective endoluminal imaging.

2.2.2 pCLE imaging in the upper gastrointestinal tract

In Barrett’s esophagus, current guidelines on surveillance recommend target biopsies for visible lesions followed by random four-quadrant biopsies every 2 cm (31). In practice, this is costly, time consuming and cumbersome to perform. Unsurprisingly, there is poor adherence to these recommendations in the UK at 41% (32) and the US at 44-56% (33). It is important to note that dysplastic or cancer changes are extremely focal and therefore may occupy small areas in a relatively large segment of Barrett’s. Random four-quadrant biopsies only allow sampling of 3.5% of the affected segment which is clearly inadequate and there are inherent concerns that biopsies might not always be taken from the site where high grade dysplasia or cancer has developed.

In light of these challenges, the use of pCLE could potential help avoid biopsies on sites deemed ‘normal’ on imaging and hence promote targeted biopsies on abnormal looking images. Furthermore, with the advent of real time in vivo, in situ diagnosis tools, clinicians now have the option of performing therapeutic procedures, i.e., endoscopic ablative therapy or mucosal resection on high grade dysplasia or intramucosal cancer in the same endoscopy session, which would otherwise be done at a repeat procedure after conventional biopsy results.
Recent work has looked into developing criteria for differentiating dysplastic from non-dysplastic segments of Barrett's. This criterion is based on changes in the architectural appearances of columnar cells and blood vessels within the mucosa. An initial study by Kiesslich et al demonstrated that Barrett’s-associated dysplasia could be predicted with a sensitivity of 93% and a specificity of 98% (34). Compared with gastric metaplasia, Barrett’s metaplasia could be predicted with sensitivity and specificity of 98% and 94%, respectively. In healthy esophagus, the columnar epithelium appears as flat cells without any crypts or villous-like appearance. The blood vessels are located within papillae. In neoplastic segments of Barrett’s, the epithelium become dark and irregularly thickened and the surrounding blood vessels become irregular. In the presence of adenocarcinoma, there is haphazard arrangement of villiform structures and crypts and dark columnar cells. Using these criteria, Pohl et al tested the diagnostic characteristics of pCLE for the detection of invisible Barrett’s-associated dysplasia (35). The overall sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 80%, 94%, 44%, and 99%, respectively, with good inter-observer agreement (Kappa=0.6) (35). Further modifications to the classification by Wallace and colleagues demonstrated that the preliminary accuracy and interobserver agreement for the detection of intraepithelial neoplasia in Barrett's has very high accuracy (91%) and almost perfect interobserver agreement (κ=0.83). This led to the development of Miami Classification thorough consensus development among international experts from USA and Europe (Figure 2.8). A recent multicentre randomised controlled trial demonstrated an increased sensitivity and specificity of 73% and 84%, respectively of detecting high grade dysplasia or early carcinoma when pCLE was used in combination with white light endoscopy or narrow band imaging (36).
<table>
<thead>
<tr>
<th>Classification</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal squamous epithelium</td>
<td>- Flat cells without crypts or villi &lt;br&gt; - Bright vessels within papillae (intra papillary capillary loops)</td>
</tr>
<tr>
<td>Non dysplastic Barrett's esophagus</td>
<td>- Uniform villiform architecture &lt;br&gt; - Columnar cells (block arrow) &lt;br&gt; - Dark &quot;goblet&quot; cells (thin arrow)</td>
</tr>
<tr>
<td>High grade dysplasia</td>
<td>- Villiform structures &lt;br&gt; - Dark, irregularly thickened epithelial borders (arrow) &lt;br&gt; - Dilated irregular vessels (block arrow)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>- Disorganized/loss of villiform structure and crypts &lt;br&gt; - Dark columnar cells (thin arrow) &lt;br&gt; - Dilated irregular vessels (block arrow)</td>
</tr>
</tbody>
</table>

Figure 2.8 Miami classification for esophageal epithelium on pCLE image acquisition [Adapted from Wallace et al, (37)]
2.2.3 pCLE imaging in the lower gastrointestinal tract

Similarly in the colon and rectum, it is well recognised that white-light endoscopic inspection alone cannot reliably distinguish between neoplastic and non-neoplastic lesions. It is therefore routine practice that all visualized lesions including small polyps are biopsied or removed during colonoscopy. However, the cumulative effect on costs of histopathology and complications are appreciable when multiple biopsies (>30) are performed during surveillance colonoscopy for ulcerative colitis (38). Furthermore, the prevalence of malignancy in small polyps are very low and over 90% of all polyps are <10 mm in diameter.

Using clinically validated diagnostic criteria, Mainz and Miami classifications (Figures 2.9), several initial eCLE and pCLE studies demonstrated that it is feasible to differentiate neoplastic from non-neoplastic mucosa of the colon and rectum with good diagnostic accuracy (28, 37, 39-41). When used in combination with narrow band imaging (NBI), the accuracy of pCLE and NBI was extremely high, approaching the accuracy of histopathology (42) and recent preliminary work by the same group reported that is also feasible to detect residual neoplasia following endomucosal resection of colorectal lesions (43). Additionally, the utilization of pCLE could potentially be considered as a supplementary tool for the marking of lateral margins of flat adenomas during transanal endoscopic microsurgery (TEMS). Whilst this has been described on eCLE imaging, the role of pCLE in ascertaining these margins warrants evaluation (44).

From the practicalities of image interpretation, there are no obvious differences in the accuracy of interpretation between pathologists and endoscopists in post-hoc (offline) studies (45) and inexperienced endoscopists were able to learn to interpret images quickly with a short learning curve (46, 47). Evidently, its role as a supplementary imaging tool for point inspection of lesions identified by red flag techniques might be an interesting application in the future. However, further large-scale studies are needed to clarify which combination of red flag technique would increase the yield of neoplasia detection. A summary of all the original studies assessing the diagnostic accuracies of pCLE on colorectal polyps are provided in Table 2.2.
<table>
<thead>
<tr>
<th>Classification</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal colon</strong></td>
<td>- Round crypt structures</td>
</tr>
<tr>
<td></td>
<td>- Dark goblet cells (arrow)</td>
</tr>
<tr>
<td></td>
<td>- Regular, narrow vessels surrounding crypts (block arrow)</td>
</tr>
<tr>
<td><strong>Hyperplastic polyp</strong></td>
<td>- Crypts with slit or stellate openings (pits)</td>
</tr>
<tr>
<td></td>
<td>- Bright non-thickened, uniform epithelium</td>
</tr>
<tr>
<td></td>
<td>- Dark &quot;goblet&quot; cells (thin arrow)</td>
</tr>
<tr>
<td></td>
<td>- Small vessels (block arrow)</td>
</tr>
<tr>
<td><strong>Adenoma</strong></td>
<td>- Irregular or vill structures (note even &quot;tubular&quot; adenoma may have villiform structure on pCLE)</td>
</tr>
<tr>
<td></td>
<td>- Dark, irregularly thickened epithelium</td>
</tr>
<tr>
<td></td>
<td>- Decreased goblet cells</td>
</tr>
<tr>
<td><strong>Adenocarcinoma</strong></td>
<td>- Disorganized villiform or lack of structure</td>
</tr>
<tr>
<td></td>
<td>- Dark, irregularly thickened epithelium (thin arrow)</td>
</tr>
<tr>
<td></td>
<td>- Dilated vessels (block arrow on H&amp;E)</td>
</tr>
</tbody>
</table>

*Figure 2.9* Miami classification for colorectal lesions on pCLE image acquisition [Adapted from Wallace et al, (37)]
<table>
<thead>
<tr>
<th>Author, year</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>No. of patients</th>
<th>Areas assessed</th>
<th>Screening method</th>
<th>System</th>
<th>Interpretation</th>
<th>Contrast agent</th>
<th>Diagnostic criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kiesslich 2004</td>
<td>98</td>
<td>99</td>
<td>N/A</td>
<td>N/A</td>
<td>45</td>
<td>390</td>
<td>CE</td>
<td>eCLE</td>
<td>N/A</td>
<td>FS</td>
<td>Mainz</td>
</tr>
<tr>
<td>Kiesslich 2007</td>
<td>95</td>
<td>98</td>
<td>N/A</td>
<td>N/A</td>
<td>153</td>
<td>134</td>
<td>CE &amp; WLE</td>
<td>eCLE</td>
<td>RT</td>
<td>FS</td>
<td>Mainz</td>
</tr>
<tr>
<td>Wang 2007</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>54</td>
<td>N/A</td>
<td>WLE</td>
<td>pCLE</td>
<td>N/A</td>
<td>FS*</td>
<td>N/A</td>
</tr>
<tr>
<td>Hurlstone 2008</td>
<td>97</td>
<td>99</td>
<td>95</td>
<td>100</td>
<td>43</td>
<td>802</td>
<td>CE</td>
<td>eCLE</td>
<td>RT</td>
<td>FS</td>
<td>Mainz</td>
</tr>
<tr>
<td>Hsiung 2008</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>WLE</td>
<td>pCLE</td>
<td>N/A</td>
<td>FS*</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Goetz 2009</td>
<td>100</td>
<td>98</td>
<td>N/A</td>
<td>N/A</td>
<td>36</td>
<td>67</td>
<td>CE</td>
<td>eCLE</td>
<td>RT</td>
<td>CV</td>
<td>Mainz</td>
</tr>
<tr>
<td>Buchner 2010</td>
<td>91</td>
<td>76</td>
<td>89</td>
<td>81</td>
<td>75</td>
<td>119</td>
<td>HD- WLE</td>
<td>pCLE</td>
<td>D</td>
<td>FS</td>
<td>Miami</td>
</tr>
<tr>
<td>Sanduleanu 2009</td>
<td>97</td>
<td>93</td>
<td>N/A</td>
<td>N/A</td>
<td>72</td>
<td>116</td>
<td>CE</td>
<td>eCLE</td>
<td>RT</td>
<td>FS</td>
<td>Mainz</td>
</tr>
<tr>
<td>Gomez 2010</td>
<td>76</td>
<td>72</td>
<td>N/A</td>
<td>N/A</td>
<td>53</td>
<td>75</td>
<td>WLE</td>
<td>pCLE</td>
<td>D</td>
<td>FS</td>
<td>Miami</td>
</tr>
<tr>
<td>De Palma 2010</td>
<td>100</td>
<td>85</td>
<td>91</td>
<td>100</td>
<td>20</td>
<td>32</td>
<td>WLE</td>
<td>pCLE</td>
<td>RT</td>
<td>FS</td>
<td>Mainz</td>
</tr>
<tr>
<td>Buchner 2011</td>
<td>81- 96</td>
<td>34- 67</td>
<td>N/A</td>
<td>N/A</td>
<td>54</td>
<td>76</td>
<td>WLE</td>
<td>pCLE</td>
<td>D</td>
<td>FS</td>
<td>Mainz</td>
</tr>
<tr>
<td>Xie 2011</td>
<td>94</td>
<td>96</td>
<td>97</td>
<td>92</td>
<td>115</td>
<td>115</td>
<td>WLE</td>
<td>eCLE</td>
<td>RT</td>
<td>FS</td>
<td>In- house¥</td>
</tr>
<tr>
<td>Gunther 2011</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>150</td>
<td>10</td>
<td>WLE; CE</td>
<td>eCLE</td>
<td>RT</td>
<td>FS</td>
<td>Mainz</td>
</tr>
<tr>
<td>Van den Broek 2011</td>
<td>65</td>
<td>82</td>
<td>N/A</td>
<td>N/A</td>
<td>22</td>
<td>135</td>
<td>NBI WLE</td>
<td>pCLE</td>
<td>D</td>
<td>FS</td>
<td>Miami</td>
</tr>
<tr>
<td>Hlavaty 2011</td>
<td>100</td>
<td>98</td>
<td>66</td>
<td>100</td>
<td>45</td>
<td>100</td>
<td>CE WLE</td>
<td>eCLE</td>
<td>N/A</td>
<td>FS</td>
<td>Mainz</td>
</tr>
<tr>
<td>Kuiper et al, 2011</td>
<td>66</td>
<td>83</td>
<td>N/A</td>
<td>N/A</td>
<td>23</td>
<td>116</td>
<td>N/A</td>
<td>pCLE</td>
<td>D</td>
<td>FS</td>
<td>In-house</td>
</tr>
<tr>
<td>Shahid et al, 2012</td>
<td>86</td>
<td>78</td>
<td>76</td>
<td>88</td>
<td>65</td>
<td>130</td>
<td>NBI</td>
<td>pCLE</td>
<td>D</td>
<td>FS</td>
<td>Miami</td>
</tr>
</tbody>
</table>

**Table 2.2** Original studies assessing the diagnostic accuracy of CLE on differentiation between neoplastic from non-neoplastic colorectal lesions. FS = Fluorescein sodium; AH = Acriflavine hydrochloride; WLE = White light endoscopy; NBI = Narrow band imaging; CE = Chromoendoscopy; RT = Real-time diagnosis; D = Delayed (offline) diagnosis.
In the era of personalised medicine, there has been increasing interest in using pCLE to visualise molecular markers of neoplastic cells. Development of targeted peptide probes and antibodies are rapidly underway and visualisation of neoplastic cells though targeted binding of these fluorescent-tagged markers have now been reported. Hsiung et al demonstrated that it is possible to visualise increased fluorescence on dysplastic human colonocytes following administration of targeted heptapeptides labelled with fluorescein (48). Similarly, preclinical work on xenograft models and human tissue specimens demonstrated that colorectal cancer cells could be visualised and differentiated by using fluorescent-tagged antibodies that binds specifically to human epidermal growth factor (49) and vascular endothelial growth factor receptors (49). These novel approaches represent a more targeted method of identifying of neoplastic cells instead of relying on morphological appearances provided by non-specific fluorescent staining.

### 2.2.4 pCLE imaging in the biliary tree

Elsewhere, initial results from a multi-centre study reported a promising role of pCLE in discriminating benign from malignant biliary strictures (50). In a disease where low survival rates are often caused by late diagnosis, these findings could potentially contribute to earlier diagnosis as current available mainstay diagnostic options for endoscopic sampling such as cytology brushing, biopsy and fine needle aspiration are limited by their low sensitivities.

The Miami classification was subsequently proposed to define the range of reticular patterns and dark bands seen on pCLE imaging (Figure 2.10) (51). Utilizing the proposed criteria for diagnosis of malignant strictures, Talreja and colleagues however reported that there was low inter-observer agreement was among the assessors (52, 53). Additionally, Loeser and colleagues suggested that a negative pCLE study of the biliary tree could potentially be used to rule out the presence of malignancy, but there are frequent false positives using the earlier classification (54). Evidently, the
higher false-positive cases were induced by benign inflammatory conditions, resulting in significantly lower test specificity (51).

In light of these, the Paris classification was proposed as a refinement of the existing Miami classification to improve the accuracy of pCLE by including a further category to distinguish benign inflammatory strictures (55) (Figure 2.11). Whilst the preliminary inter-observer agreement findings were encouraging (56), it is evident that prospective multicentre studies are needed to further validate this refined classification criteria. A summary of all the original articles published to date on pCLE imaging of indeterminate biliary strictures is shown in Table 2.3.

The diagnostic challenge in malignant tumours of the biliary tree pertains to poor sensitivity of tissue sampling techniques during ERCP. Unlike the aforementioned applications in the gastrointestinal tract where tissue sampling usually provides definitive diagnosis, it appears that pCLE could have the potential to overcome challenges associated with histological diagnosis. The clinical argument here no longer pertains to “to biopsy or not to biopsy”, instead the cellular information provided may facilitate the decision-making process pertaining to definitive management of these patients.

<table>
<thead>
<tr>
<th>Author, year, ref</th>
<th>No. of patients</th>
<th>Malignant strictures</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meining, 2009,</td>
<td>14</td>
<td>6</td>
<td>83</td>
<td>88</td>
<td>86</td>
</tr>
<tr>
<td>Loeser, 2011, (54)</td>
<td>14</td>
<td>6</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Giovannini, 2011, (57)</td>
<td>37</td>
<td>23</td>
<td>83</td>
<td>75</td>
<td>86</td>
</tr>
<tr>
<td>Shieh, 2011,(58)</td>
<td>11</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Othman and Wallace, 2011,(59)</td>
<td>89</td>
<td>40</td>
<td>98</td>
<td>67</td>
<td>81</td>
</tr>
<tr>
<td>Meining, 2011, (50)</td>
<td>102</td>
<td>40</td>
<td>98</td>
<td>67</td>
<td>81</td>
</tr>
<tr>
<td>Talreja, 2012 (52)</td>
<td>N/A</td>
<td>25</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.3 Original articles assessing the diagnostic accuracy of pCLE on differentiation between benign and malignant biliary strictures
Figure 2.10 Miami classification of the biliary tract from pCLE images [Adapted from Meining et al, (51)]

Figure 2.11 The additional of pCLE description of inflammatory stenosis forms the Paris classification, a modification of the Miami classification [Adapted from Meining et al, (55)]
2.2.5 pCLE imaging in the urinary tract

Similar to gastrointestinal tract applications, detection of neoplastic tissues during cystoscopy relies on white light imaging for accuracy and comprehensiveness. The pivotal shortcomings of white light cystoscopy include that of flat lesion detection, real-time in situ tumor delineation to enable complete resection, differentiation between inflammatory changes from that of malignancy, and determination of the grade and stage of tumour (60, 61). Whilst the role of pCLE might be limited for the latter application, the rest are pertinent to the sub-surface imaging characteristics conferred by pCLE.

The feasibility of using pCLE to detect histological differences between normal and neoplastic urothelium on acriflavine-stained, excised specimens were first described by Sonn and colleagues (62). Further in vivo validation studies confirmed that pCLE image acquisition of benign and neoplastic tissues were feasible in patients undergoing TURBT and nephrectomy (63) (Figure 2.12). Additionally, the interobserver agreement and diagnostic accuracies of neoplastic bladder pCLE images were comparable to that of WLC alone with a corresponding moderate inter-observer agreement (64). As with pCLE imaging of the biliary tree, its role in improving bladder cancer detection and completeness of tumour resection during transurethral procedures is still under evaluation. It is envisaged that a concurrent red flag technique is required to facilitate targeted imaging with pCLE.
Figure 2.12 pCLE images of (A) normal bladder urothelium depicting an umbrella cell layer: note the characteristic large polygonal-shaped cells; (B) Low grade urothelial carcinoma with crowding of uniform-appearing cells; (C) Fibrovascular stalk with a thickened endothelial layer; (D) Cross-sectional view of the fibrovascular stalk with erythrocytes in the vascular core; (E) High-grade urothelial carcinoma with pleomorphic and distorted sheet of cells. (F) Distorted fibrovascular stalk with variation in vascular cores sizes. [Adapted from Wu et al, (63)]

2.2.6 pCLE (nCLE) imaging of pancreatic lesions

Endoscopic ultrasound guided fine needle aspiration (EUS-FNA) is a commonly performed procedure to assess cystic and solid lesions of the pancreas (65). However, that is frequently complicated by sampling errors, non-diagnostic cytology and limited on-site cytological services (66, 67). Multiple FNA passes are frequently performed to obtain diagnostic material (67). These are time-consuming and increase the risk of complications such as pancreatitis. It is envisaged that the use of pCLE could
potentially reduce the need to rely on EUS-FNA procedures as provision of real-time microscopic information could potentially provide diagnostic material safely and quickly. Utilizing a needle-based CLE (nCLE) probe that is compatible with a 19-gauge FNA needle, image acquisition were feasibly acquired under ultrasound guidance with the presence of epithelial villous structures on nCLE images being consistent that of neoplastic tissues (68, 69) (Figure 2.13). Preliminary data suggests that nCLE has a high specificity in the detection of PCN, but limited by a low sensitivity (68, 69). The safety of nCLE still requires further evaluation as a few patients developed post-procedure pancreatitis. Much work needs to be carried out to validate these findings and standardise feature descriptions.

Figure 2.13 nCLE images of normal pancreatic tissue and intraductal papillary mucinous neoplasam. (A) Normal pancreatic tissue depicts thin dark bands with reticular pattern. (B-C) Histology images of tumor tissue cystic dilatation of ducts with papillary proliferation. (D-E) nCLE images of the tumor with papillary projections. [Adapted from Konda et al, (68)]
2.27 pCLE imaging of the respiratory tract

Utilizing a pCLE miniprobe adapted for respiratory airways (1-mm flexible Alveoflex® miniprobe), several studies reported that both elastic fibres and macrophages were readily visible in the alveolar wall (26, 70-74). The latter were seen to be positively correlated with the number of cigarettes smoked per day (70). The presence of marked emphysema in patients with chronic obstructive pulmonary disease was depicted on pCLE images as loss of elastic walls, increased spacing between septal walls, loss of fluorescence from bullae and a subsequent reticular pleural image (26) (Figure 2.14). Conversely, the elastic fibres were reported to be thickened, rigid and in high concentration in patients with interstitial lung diseases (74) as compared to the thin and mobile fibres seen in healthy alveoli. The addition of intravenous FS resulted in overfluorescence of the alveolar wall and did not appear to depict any additional morphological features (26).

A recent study by Fuchs et al shed some light on the potential role of pCLE in lung cancer diagnosis. In 32 patients that underwent bronchoscopy for suspected lung malignancies, neoplastic and non-neoplastic features of respiratory epithelial lining on pCLE images could be predicted with high accuracy (sensitivity 96.0%, specificity 87.1%, accuracy 91.0%) when AH was applied topically as the contrast agent (29). Whilst this may potentially enable the rapid diagnosis of neoplasia during bronchoscopy, the relevance of these findings remain questionable as tissue biopsy from an obvious tumour is still warranted for definitive histological evaluation. Whilst transbronchial biopsies of the respiratory epithelium have its own aforementioned risks, the extent of biopsies required are not comparable to that of during surveillance of Barrett’s esophagus and ulcerative colitis. More importantly, the risks and benefits of obviating the need for biopsies on the basis of pCLE images in a patient with risk factors warrant a systematic evaluation before its potential benefits could be reliably ascertained.
Figure 2.14 pCLE images of disrupted elastic fibres (yellow circles) (A-C) with patients with lung emphysema. Image D depicts the “snapping” of elastic fibres on consecutive still pCLE images. On the background, fluorescent blobs of macrophages were visualised which is consistent with chronic tobacco smoking. [Adapted from Newton et al, (26)]

2.3 Challenges associated with pCLE imaging

2.3.1 Clinical challenges with envisaged applications

Since the advent of pCLE in 2007, publications pertaining to pCLE images obtained from a variety of tissues were akin to that of ‘mushrooms after the spring rain’. Whilst the plethora of research composed of several promising high quality feasibility studies such as those pertaining to the surveillance of BE and assessment of indeterminate biliary strictures, much of it lacked thorough and rigorous evaluation on the practicality of its intended clinical role. Unlike traditional biopsies where bleeding occurs at sampling sites, the lack of visual cues to localise the sites where pCLE assessment was performed can be challenging. Given that the envisaged role of pCLE in surveillance of BE is to guide the need for biopsies, it is therefore important that the sites where biopsies are performed
correlate precisely to that of the sites assessed by pCLE imaging. The Barrett’s segment presents a
unique challenge in that it could appear homogenously inflamed thereby lacking essential visual cues
to facilitate precise localization of these sites. Evidently, the absence of bleeding points combined with
tissue deformation secondary to peristalsis and intraluminal pressure changes could further impede the
accuracy of localization. Consequently, this could lead to pCLE and biopsy assessment mismatch. The
implications of this discordance could lead to diagnostic dilemma, inconsistent management and
treatment for BE patients.

On another note, it is important to recognise that all pCLE studies pertaining to BE to date relied to the
yield of biopsy samples of suspicious lesions and four-quadrant random locations to determine the
presence or absence of high grade dysplasia or carcinoma (35-37). It is obvious that this study design
would have underdiagnosed its true prevalence in the study population. Therefore, the evidence
gathered could only suggest that there might be a role for pCLE in reducing the need for biopsies
during random sampling and from that of suspicious lesions. There is no evidence to date to suggest
that it would increase detection rates of high grade dysplasia or carcinoma from a relatively
inconspicuous Barrett’s region. These challenges are pertinent to that of lower gastrointestinal tract
surveillance for ulcerative colitis. Whilst numerous studies have shown high diagnostic accuracies for
neoplastic and non-neoplastic morphology on pCLE images, there is no evidence to suggest that the
use of pCLE increases the overall detection rates of neoplasia (42, 47, 75). It reaffirms that the
effectiveness of pCLE at reducing the need for biopsies for both upper and lower gastrointestinal tract
applications is largely dependent on the ability of concurrent red flag imaging modalities such as
narrow band imaging (NBI) and chromoendoscopy (CE) to facilitate targeted pCLE assessments.
However unlike that of the Barrett’s segment, large colorectal polyps of more than 10 mm in diameter
harbour a higher risk of neoplastic transformation as compared that of smaller polyps and thereby
warrant excision for definitive histology (76). It is unlikely that pCLE assessment of large polyps
would confer any advantage as it would not impact decision-making. On another note, hyperplastic
polyps are known traditionally to be non-dysplastic (77, 78) and have little potential for malignant
transformation. However, recent evidence showed that serrated variants with similar morphological
architecture to that of hyperplasia harbours malignant potential (79, 80). The discrimination between hyperplastic and sessile serrated polyp, the latter a well-recognized precursor to malignancy, has shown to be highly variable on histology, even among experienced pathologists (81). It remains doubtful whether pCLE imaging could project these subtle differences in architectural variation given it is already hugely challenging on conventional histology.

Similarly, the challenges associated with localization of neoplastic sites are pertinent to pCLE urology applications. Approximately 30-44% of patients that underwent transurethral resection of bladder tumour (TURBT) for non-muscle invasive bladder cancer were found to have recurrence within 2-8 weeks following the procedure (60). This is commonly attributed to the inherent inability to visualize synchronous neoplastic sites during white light cystoscopy (WLC) which evidently has sensitivities and specificities ranging from 62-84% and 43-98%, respectively (82). Emerging red flag techniques such as fluorescence cystoscopy (61) and NBI (83, 84) have shown promising potential to facilitate detection of these neoplastic sites but are hampered by its high false positive rates (85-87). There might be a role of pCLE to provide in situ confirmation of neoplastic morphology on these sites thereby facilitating targeted biopsies. Needless to say, its role is dependent on the ability of the aforementioned red flag techniques to identify the ‘needle in the haystack’ in the first place.

The potential role of pCLE for assessment of indeterminate biliary strictures arguably holds one of the most exciting and promising of all envisaged clinical applications. Distinguishing between benign and malignant strictures is difficult, with 5.2 to 24.5 per cent of these found to be benign after histological examination of the resected specimens (88). Given that the diagnostic yield of multi-modality imaging with CT scan, MRCP, EUS, ERCP and biliary cytology is so low, any modest improvement in diagnostic accuracy conferred by pCLE would translate into a major improvement in the definitive management of these patients. However compared with the initial classification proposed in 2011 (50), Loeser et al reported that the presence of dilated blood vessels were not specific for malignancy as these were present on pCLE images from both normal CBD and malignant strictures (54). These vessel characteristics are still being used as one of the key features for malignancy in revised
classifications (51, 56). The low interobserver agreements reported by Talreja et al are a cause for concern (52). Additionally, there appears to be a lack of understanding on the histological meaning of the imaging patterns observed. It is evident that the current data is still sparse and consequently the diagnostic criteria are still evolving. On another note, whilst the smaller pCLE miniprobe size confers more space for angulation of the probe tip towards stricture surface, it does come at the expense of image quality and spatial resolution (58). The trade-off between optical resolution and physical deployment warrants evaluation to ensure that a compromise is achieved that allows both image acquisition and interpretability of its images feasible.

The role of pCLE in the respiratory tract remains unclear at present. A significant amount of diagnostic information is lost on pCLE imaging (26) and the structural changes visualised in patients with emphysema and interstitial lung diseases are non-specific findings. These conditions are diagnosed on clinical grounds and high resolution CT scan; therefore pCLE imaging might not be warranted. Whilst Fuchs reported that pCLE neoplastic changes could be discerned when AH was utilized (29), the clinical significance of these findings is unclear as biopsies would be required for definitive diagnosis. The origin of these neoplastic lesions could be primary or secondary, and both hold important prognostic information. Whilst it is recognised that transbronchial biopsies carry a small risk of pneumothorax, the requirement for multiple biopsies does not approximate to that of Barrett’s and ulcerative colitis surveillance.

At present, there is still paucity of information pertaining to pCLE imaging of pancreatic cysts. The two studies published to date describe the safety and feasibility of the procedure (68, 69). The preliminary findings reported warrants further validation. It remains unclear whether pCLE images obtained from a single assessment site are representative of the entire cyst. The field of view conferred by AQ-Flex® pCLE miniprobe is relatively small (280 x 280 µm) and mosaics could not be constructed. Multiple imaging attempts at different sites might be required to provide representative images of the cysts. Additionally, the leakage of fluorescein from the puncture sites could potentially
impede visualization of morphology. These are some technical considerations that warrant systematic evaluations.

2.3.2 pCLE image interpretation and miniprobe stability

One of the challenges common to pCLE image acquisition across a range of clinical applications is the clinicians’ ability to obtain high quality and consistent in vivo, in situ images. It requires slow and controlled movements, often over a few millimetres, for large area surveillance. This is a challenging task in the presence of bowel peristalsis and cardiorespiratory movements. In gastrointestinal endoscopy, this requires the endoscopists to manipulate its wheels or change the direction of its torque. Variations in the intraluminal insufflation pressures can lead to unintended deflation of the lumen. Whilst this might not be appreciable from the endoscopic view, these motions can distort the quality of real-time mosaics. The mobility of the tissue of interest can pose an additional challenge especially in cases of pedunculated polyps of the colon. Protruding polyps are difficult to stabilise as it tend to flip over the tip of the confocal endoscope upon contact. The reported rates of failure to assess protruding polyps using eCLE can be as high as 57% with difficulty in stabilising polyps cited as the main contributing factor (89). A more recent study by Kuiper et al demonstrated that nearly a quarter of pCLE videos obtained during colonoscopy failed to demonstrate any crypts or vessels. This corresponded to almost 12% of lesions encountered and much of the problems encountered were difficulty stabilising the tip probe against the cohort of mobile lesions (90).

These challenges are not confined to intraluminal imaging but to all solid organ and soft tissue pCLE imaging. The effect is magnified on applications in close proximity to the torso and neck where respiratory movements of the chest wall and strap muscles can lead to significant distortions during pCLE image acquisition. Rosa et al reported that these movements can be of several centimetres of magnitude which could lead to unstable images and inability to create meaningful mosaics (91). To reduce the effects of tissue deformation and movements associated with cardiorespiratory and bowel movements during in vivo pCLE imaging, the use of closed-loop, force sensitive robotic probes has
been proposed (92). The principle behind this is to maintain a desired contact force between the tissue surface and tip of the pCLE probe for image consistency. Using these smart instruments, predetermined contact forces which compensates for physiological movements of tissue and involuntary movements of the clinician’s hand have been shown to improve the consistency of images obtained as evidenced by minimal crypt translation on still images of bowel tissues obtained at per second interval. Initial in vivo studies on porcine rectal mucosa reported that pCLE images could be obtained at contact forces as low as 100 mN (93). Utilization of smart surgical instruments that reduce hand-held tremor or compensate for physiological movements could be pertinent to surgical applications of pCLE (92). More recently, the use of industrial lightweight robots such as KUKA as a means for controlling pCLE miniprobe navigation on simulated solid organs and cavity-based models have been investigated (94-96). Whilst the robustness of utilizing this method has been tested and preliminary results demonstrated that reconstruction large mosaics with accurate spatio-localization is possible, it remains to be seen whether integration of such approach would be feasible and thereby warrants further validation in clinical studies.

2.4 Role of pCLE imaging in surgery

2.4.1 Overview

The role of pCLE outwith the endoluminal environment for surgical applications has been relatively unexplored. In particular, there is little reported on its ability to characterise morphological features beyond mucosal or luminal epithelium. This is unsurprising given that the pCLE miniprobes are currently CE-marked for clinical applications within the endoluminal tracts. Additionally, this is partly attributed to the commercially driven strategy to develop applications within the endoluminal tract given the miniprobe’s unique physical and optical characteristics.
Most studies published to date consist of proof of concept work or feasibility studies on animal models and humans. A study by Newton et al reported the feasibility of utilizing a flexible access robot known as i-Snake® as a potential delivery tool to aid stable pCLE image acquisition of solid organs in the peritoneal cavity (Figure 2.15) (97). Morphological images from liver subsurface parenchyma, splenic subcapsular vessels and peritoneal lining were readily visualized. Similarly, studies by Goetz and colleagues reported the use of an in-house designed rigid CLE to image the liver microarchitecture during mini-laparoscopy (98, 99). The majority of publications on in vivo CLE imaging were pertinent to neurosurgery where completeness of excision of brain tumor and preservation of normal brain tissue is pivotal to overall morbidity and mortality of these patients (100, 101). Neoplastic and non-neoplastic pCLE features of brain tissues were visualised and demonstrated good correlation with respective histology (30, 102-104) (Figure 2.16). However, the work described to date still revolves around the feasibility of in situ CLE imaging and evidence pertaining to its impact on achieving tumor-free resection margins or the overall morbidity in these patients is yet to be established.

There were suggestions that pCLE could potentially be used to guide tumor resection margins during TEMS (44) and TURBT procedures (63, 105). Hence, its diagnostic potential during routine endoscopy could potentially be extrapolated to applications pertaining to endoscopic-based surgery. Additionally, it could also be used to confirm complete removal of neoplastic tissues on the lateral margins of mucosal defect created from surgery. To date, no clinical studies have evaluated the potential role of pCLE in these applications.

It is evident that the role of pCLE as a potential imaging adjunct during surgery is still in its infancy. With the exception of neurosurgery applications, much of the published work has no clear clinical applications in mind and there is an obvious paucity of clinically driven pCLE research towards intraoperative use. The provision of real-time information of the tissues encountered during surgery i.e. tissue tumor-free status, tissue type confirmation or tissue viability/vascularity, depending on the intended application, could potentially have a far greater clinical impact on the outcome of the
operation. Evidently, these information could be used to guide real-time surgical decision-making and the potential clinical applications in this unexplored surgical avenue warrants investigation.

Figure 2.15 (A & B) A laparoscopic view of transvaginally inserted flexible robot i-Snake® performing retroflexion in the pelvis. (C) Tip of the pCLE probe deployed against the liver, (D) spleen and (E) peritoneum (F) pCLE image of liver hepatocytes (G) pCLE image of splenic cords of Billroth; (H) Parietal peritoneum of the abdominal wall with the arrow depicting a blood vessel. [Adapted from Newton et al, (97)]
Figure 2.16 System set-up of rigid CLE probe and AH-stained human brain tissue CLE morphology (A) The rigid CLE has a shaft diameter of 7mm. (B) Human brain tissues containing normal and adjacent tumor section. (C-D) Normal brain tissue with neuron or microglial cells with appendices (arrows). (E-F) Tumor tissues reveals excessive growth with atypic nuclei and abnormal nuclear-to-cytoplasm ratio. [Adapted from Foersch et al (30)].

2.4.2 Potential applications in breast and endocrine surgery

It is evident that the role of pCLE as a potential imaging tool to facilitate intraoperative decision-making is predicated on the following criteria:

- An intraoperative problem that is currently unaddressed by current techniques / technologies
- Information provided will have an impact on real-time intraoperative decision-making
• Time-efficient image acquisition and interpretation by the surgeon in the operating theatre

Taking these into account, we envisage that pCLE might have a promising role as an intraoperative imaging device in the following surgical applications (Figure 2.17):

• Detection of residual disease on the cavity walls during breast conserving surgery (BCS) / wide local excision (WLE) for breast cancer
• Differentiation between parathyroid from non-parathyroid tissues during parathyroidectomy for primary and secondary hyperparathyroidism
• Assessment of viability status of preserved parathyroid glands during total or completion thyroidectomy for benign and malignant conditions

Over the last 30 years, advances in mammographic screening have led to an increase in the diagnosis of small, non-palpable breast cancers (106). As a result, breast conserving surgery (BCS), which involves removal of the cancer together with a surrounding cuff of normal breast tissue, has become the standard intervention and is generally regarded as sufficient curative treatment in appropriately selected patients (107).

In BCS, it is important to achieve an optimal balance between ensuring complete removal of breast cancer and preservation of tissues for good cosmesis (108). The former is generally assessed through histology on the margins of the excised tissue (109). Positive surgical margins (the presence of tumor cells at the edge of excised tissue) are associated with a high risk of developing local recurrence and so the patient will often be required to return to theatre for a reoperation (110-112). This carries a further risk of postoperative infections (113), has a negative impact on cosmesis (114), and increases costs due to longer stays in hospital (114). At present, the proportion of patients with positive surgical margins ranges from 20% to 40% and the national reoperation rates are 30% for non-invasive and 18% for invasive breast cancer (114, 115).

It is evident that the completeness of tumor removal rates achieved during WLE warrants improvement. There is increasing evidence that the tumour bed status is an independent predictor of
local recurrence (116, 117). Systematic cavity shaves were shown to reduce re-excision rates following BCS (118). Hence, the role for pCLE could potentially reside on visualization of residual foci of tumor cells that were inadvertently left behind on the cavity walls following WLE. Equipped with these information, the surgeon could potentially decide whether further excision of the corresponding cavity wall is needed based on real-time information provided by pCLE assessment. This will avoid the need for a reoperation as further excision, guided by pCLE, could be performed at the time of the index surgery.

Figure 2.17 Venn diagram depicting the envisaged intraoperative pCLE application and subsequent decision-making processes.

Parathyroid glands (PG) on the other hand are notoriously difficult to identify during surgery because of their small size and appearance that is often similar to lymph nodes, fat, and occasionally thyroid tissue (119). Even in the hands of an experienced surgeon, this could be challenging especially in reoperative cases (120) and multinodular goitres where tiny superficial nodules could mimic parathyroid glands (121). The sensitivities of the gold standard imaging modalities such as ultrasound
(USS) and sesitimibi scans (SS) are far from perfect, with rates of detecting disease PGs ranging between 51 to 96% and 34 to 100%, respectively (122, 123). Both their sensitivities decrease even further in the presence of multiglandular disease (MGD) (124, 125). Failure to localise PG preoperatively warrants bilateral neck exploration to identify the diseased gland (126). The location of these glands could vary significantly, especially the inferior PG (127). Whilst intraoperative parathyroid hormone assay (ioPTH) has a role in confirming the removal of the diseased PG (128), it still requires the surgeon to identify these in the first place.

This leads us to question whether is there a role for pCLE to help differentiate parathyroid from non-parathyroid tissues? If so, could it provide the surgeon a recourse when equivocal tissues are encountered at the time of surgery? This could obviate the need for current techniques such as frozen section analysis which is time consuming and unreliable (129). It may also provide valuable information when ioPTH is inconclusive (130). The latter is less reliable when multiglandular disease and double adenomas are present. At this point of time, the role of pCLE in identification of PGs has yet to be evaluated. A systematic search performed in November 2013 on registered clinical trial databases (National Institutes of Health, Clinical Trials.gov, European Union Clinical Trials Register) showed no current or planned studies addressing similar hypotheses.

One of the most common complications following total or completion thyroidectomy is postoperative hypocalcaemia (131). Whilst these could easily be managed with calcium and vitamin D analogue supplementations (132), permanent hypocalcaemia secondary to inadvertent removal or injury to the PG vascular supply could potentially lead to life-long debilitating complications (133). It is common practice to routinely identify at least one PG during surgery and ascertain its viability status. If the PG appeared discoloured or its vascular supply was obliterated, the surgeon would normally autotransplant this PG onto a neighbouring muscle, most commonly the sternocleidomastoids, to preserve its function (132). However, the absence of tissue discolouration is not a reliable indicator of PG viability (134) and conversely, the presence of PG discolouration do not confer necessarily represent permanent loss of PG function (135). Consequently, visual assessment of PG discoloration
as a predictor of viability is unreliable and decisions pertaining to autotransplantation of PGs remains controversial and rely heavily on the surgeon’s experience. Unsurprisingly, 24.9% of patients that underwent total thyroidectomy in the UK developed post-operative hypoparathyroidism (136). More worryingly, approximately 12% developed permanent hypocalcaemia (136).

It is well recognised that the sub-surface vasculature of gastrointestinal, biliary and urological tract tissues were readily discernible on pCLE images (Figure 2.18) (37, 137). Most of the descriptions on vascular morphology of neoplastic tissues were in pertinence to its general architecture i.e. vessel thickness, distortion and dilatation; and visualization of individual erythrocytes. If similar vascular characteristics could be visualised on PGs, we could potentially elicit valuable information on PG vascular supply and viability. Once again, this is an interesting avenue that has yet to be systematically investigated. We hypothesis that subcapsular and superficial parenchymal vasculature could potentially be visualised on pCLE imaging and that information pertinent to the presence, adequacy and quality of blood flow could be deduced. With that in mind, decisions pertaining to autotransplantation of PG could potentially be facilitated by findings on pCLE images. Chapter 6 will provide detailed descriptions of our evaluations utilising pCLE on preserved PGs.

![Figure 2.18](image-url) pCLE image depicting blood vessels (red arrows) with intraluminal erythrocytes (dark particles) on (A) Barrett’s segment; (B) Normal bladder mucosa; (C) Normal bowel mucosa. [Image A adapted from Konda et al (138), Image B provided by Dr. Joseph Liao, Stanford University, CA, USA]
2.4.3 pCLE imaging of blood-stained tissues during surgery

One of the main concerns pertinent to pCLE imaging during surgery is the effect of blood on pCLE image acquisition. Evidently, a bloodless surgical field is impossible to achieve during breast, thyroid and parathyroid surgery as these tissues are well vascularised, thus minor oozing from capillaries or small venous tributaries will occur during dissection. Most of these will be either be removed either by suction or gently dabbing with a swab after adequate haemostasis is achieved. Despite that, it is inevitable that dried blood stains will still remain on the tissue surface. Most pCLE imaging described in the literature to date were performed on relatively unperturbed epithelial surfaces with intact vasculature. The impact of blood-stained tissues on pCLE imaging of tissue architecture warrants evaluation before any aforementioned potential clinical applications is evaluated.

To this end, a feasibility study was carried out on a live, anaesthesied porcine model to test the hypothesis that pCLE could be used to visualise soft tissue morphology in an intraoperative field. Under the study protocol approved by the Home Office (No. 80/2297), a neck dissection was performed on an anaesthetised six-month-old white Landrace crossbreed female pig (70kg). The neck was chosen as our anatomical region of interest because it is relevant to one of our clinical applications of interest. The porcine models available in our animal research facility had immature mammary glands and thereby inappropriate for us to perform a realistic cavity dissection. Additionally, the thyroid gland is a highly vascularised structure and minor oozing from surrounding vasculature would provide a realistic simulation to that of the breast.

The neck dissection was performed by two surgeons (T.P.C., K.S.) who held valid personal and project licences for this study. A vertical midline incision was performed and the strap muscles, thymus, thyroid, carotid artery, internal jugular vein, trachea and esophagus carefully dissected and the respective vascular pedicle / vasculature of thyroid and thymus preserved (Figure 2.19). Haemostasis was achieved using bipolar diathermy. Following that, five mls of intravenous fluorescein sodium (FS) 10% (Martindale’s, UK) was administered as tissue contrast and the pCLE miniprobe (2.6-mm
diameter Ultra-Mini-OTM) was deployed in-situ. The morphological architecture of corresponding soft tissues are described in Figure 2.20.

**Figure 2.19** Intraoperative images of anatomical neck dissection in a porcine model. (A) Vertical neck incision was performed; (B) Self retractors were placed and a suture sling was used to retract the strap muscle. (C) Thyroid gland with a pCLE Mini-O miniprobe deployed perpendicular to its surface for image acquisition. Additional pCLE imaging was performed on the thymus (black arrow), trachea (yellow arrow), strap muscles (arrowhead) and (D) on the internal jugular vein (black arrow) and carotid artery (yellow arrow).
Figure 2.20 Real-time pCLE image mosaics of soft tissue morphology were readily discernible. (A) Polygonal shaped fat cells; (B) Cylindrical shaped longitudinal muscle cells of the esophagus; (C) striated cells of strap muscles; (D) Follicular cells of the thyroid gland using topical application of 0.05% AH as an additional contrast agent; (E) Fluorescent-stained thyroid follicles; (F) Opaque appearance of colloid in the central lumen of thyroid follicle; (G) Thymus lobule with a surrounding vascular septa.

It was evident that morphological architecture of most soft tissues within the intraoperative field of the neck could be visualised in real time using pCLE, hence suggesting that image acquisition of tissue morphology is not just limited to the epithelial linings of the gastrointestinal tract, biliary tree, urological tract or lung, respectively. The respective tissues imaged were mildly stained with dried blood on its surface and that did not impede visualization of morphological architecture throughout our imaging sessions. However, we do not envisage that imaging would be possible in the presence of active bleeding as the resulting emissions from tissues would be absorbed by the intervening uncoagulated blood between the probe tip and tissue surface. Nevertheless utilizing a sterile swab to gently dap away any liquid blood or tissue debris would suffice and pCLE image acquisition should be fairly unperturbed.

It is important to note that the timing of intravenous FS may play an important role on pCLE image acquisition of any tissues in the operative field. If FS is administered too early, any subsequent
haemorrhage would render these tissues overfluorescent. Given that the systemic dissemination of FS occurs within minutes of administration, it is envisaged that any time period following excision of the breast tumor, thyroid or parathyroid gland would suffice provided adequate haemostasis has been achieved. We envisage the same principles apply when using AH as the topical fluorescent agent. However, the latter would require a generous rinsing or washout with warm saline after topical application to remove any excess unbound fluorescence.

2.4.4 pCLE imaging through a sterile transparent sheath

The sterility of pCLE miniprobes is of prime importance when it is deployed against the tissue surface. At present, the AQ-Flex® miniprobe is the only commercial pCLE miniprobe that is CE-marked for sterilization by STERRAD® Sterilization System (139). All other pCLE miniprobes are currently not sterile and therefore may not be suitable for intraoperative deployment in a sterile surgical field. Whilst the AQ-Flex® miniprobe meets the strict sterility requirements, its physical properties may not be suitable for use as a hand-held probe. It has a relatively thin diameter (approximately 1.2mm) and its fibrebundle is not sufficiently rigid on its distal end to withstand perpendicular pressures and tissue surface frictions from lateral miniprobe movements. Consequently, construction of pCLE image mosaics might be difficult to orchestrate.

One of the most common methods to overcome this problem is to use a sterile transparent drape as a sheath to maintain sterility in the operating field. Evidently, equipments such as the gamma probe detector for sentinel lymph node biopsies (140) and nerve monitoring devices for thyroid surgery (141) have benefited from this safe and cost effective approach. As part of our initial assessment on pCLE imaging towards surgical applications, we conducted a pilot study on five freshly excised parathyroid tissues. The aim is to determine the ability of pCLE to image morphological architecture of tissues with and without a sterile transparent drape (Clinicon®, Bristol, UK) as an external sheath.
The overall system set-up and imaging results are described in Figure 2.21.

Figure 2.21 pCLE equipment and sterile transparent drape set-up. (A) The entire miniprobe is sheathed with the drape. The distal end of the drape is folded backwards and the small opening is obliterated with the blue tape. (B) The blue tape is secured around the fibrebundle so that interface between probe tip and sheath is taut. (C) pCLE image acquisition is performed utilising 2.6-mm diameter Ultra-Mini-O\textsuperscript{TM} miniprobe. (D) pCLE image mosaic of parathyroid cells with the sterile drape. (E) pCLE image mosaic without the drape.
Based on the results of this feasibility study, it is evident that the individual nuclei of parathyroid parenchyma were readily visualised through the transparent drape. Equally, the depth of imaging was not compromised as the thickness of the drape is approximately 8-10 µm. No movement artefacts were noted throughout image acquisition. The contrast between the nuclei and background stroma appeared enhanced when the drape was used. Although this is unlikely to be of any clinical significance, the smooth interface conferred by the drape allowed translation of the probe tip onto the tissue surface relatively unperturbed. This could potentially facilitate creation of longer and meaningful mosaics. Importantly, we have demonstrated that utilization of a sterile transparent drape did not impede pCLE image acquisition and that the quality of images obtained were comparable to that of without the drape. This finding forms the basis for using a sterile-draped pCLE probe for imaging vascular morphology of parathyroid glands in Chapter 6 and the future work of Chapter 5.

2.4.5 pCLE image interpretation by surgeons

Little is known about the ability of surgeons to learn the basics of pCLE image interpretation and the training materials that are required. The training curriculum of the modern day surgeon rarely involve interpretation of histology slides. Whilst there is evidence that gastroenterologists were able to acquire pCLE image interpretation skills with a relatively short learning curve (142, 143), we are mindful that most of these studies originated from centres with vast amount of pCLE clinical experience and that received commercial funding for the development of pCLE applications.

Since our aim is to evaluate the feasibility of utilizing pCLE as a potential imaging tool for surgical applications, it is prudent to establish whether surgeons who had no prior experience on pCLE imaging were able to learn the basics of pattern recognition. To that end, we developed a 15-minute in-house video tutorial that provides detailed descriptions about Miami classification of colorectal lesions (142) and assess the ability of 10 surgeons to recognise mucosal crypts and vessel architecture of neoplastic and non-neoplastic pCLE images (Figure 2.22). Further training were provided to differentiate normal from hyperplasia and adenoma from adenocarcinoma images. Following that,
participants were given a practice session that consists of 15 pCLE images followed by a question and answer session. Subsequently, all participants were then shown 50 colorectal pCLE video images* blinded to histopathology results (25 neoplastic and 25 non-neoplastic) and accuracy of interpretations were assessed.

The overall accuracy for differentiation between pCLE neoplastic and non-neoplastic images was 91.4% (range 82–96%) and interobserver agreement was 'substantial' (κ=0.7). Accuracy for the overall group was 87% for videos 1-10, 97% for videos 11-20, 97% for videos 21-30, 83% for videos 31-40 and 93% for videos 41-50. The accuracy for specific diagnostic entities (normal vs hyperplasia vs adenoma vs adenocarcinoma) was 77.4% (range 70–84%). The accuracy rates were not significantly different with age, or by clinical or endoscopy experience.

It is evident that utilizing a systematic and structured approach, surgeons with no prior experience on pCLE image interpretation were able to learn the basics of pattern recognition training. Importantly, the clinical background i.e. lack of histopathology experience did not perturb the confidence of our participants. Most felt that the key features were clearly described and easily retained. In any effort to minimise any bias, we chose to use a video tutorial as a training tool so that delivery of the learning materials were standardised to all participants. Given that each participant assessed the pCLE images independently, there were no bias from peer pressure. We chose to use pCLE images from the lower gastrointestinal tract because it was one of the few applications that had a classification that was internationally validated with excellent inter-observer agreement.
Figure 2.22 Screenshots of a video tutorial designed to train inexperienced surgeons on Miami classification of neoplastic and non-neoplastic colorectal lesions
Chapter 3: Evaluation of Acriflavine-stained Breast Morphology on pCLE Images

3.1 Introduction

3.1.1 Positive margins in breast conserving surgery

Breast cancer is the second most common newly diagnosed cancer and second leading cause of cancer death among women in the US and Europe. Breast conserving surgery (BCS) has become the ‘gold standard’ intervention for patients with smaller sized tumors and is generally regarded as sufficiently curative provided the tumor is excised with a margin of healthy breast tissue. Local recurrence following BCS is influenced by the patient’s age, tumor size and grade, the presence of multifocal or multicentric disease, and margin status (110, 112, 144, 145). Of these, the strongest predictor of local recurrence is margin status (115, 146, 147). Positive margins (disease at the edge of excised tissues) following BCS is a common indication for re-excision and it remains a significant problem with published rates varying between 15-50% (148-150). Re-excision rate estimates in the US following failed attempts at BCS ranges between 30 to 60%. Similarly in England and Wales, re-excisions are unacceptably high at 28% for non-invasive and 18% for invasive breast cancer (114). Re-excision leads to unnecessary anxiety, delays in delivery of adjuvant therapy, poor cosmesis and is cost inefficient (111).

3.1.2 Challenges with margin assessment

Whilst the impact of a positive margin on the risk of local recurrence is understood (115), the same cannot be said of ‘close’ margins. The lack of any universal consensus on what constitutes a ‘close’ margin and hence appropriate surgical management is reflected by the substantial surgeon and institutional variation observed in re-excision rates (151). Where cavity re-excision is performed for
'close’ margins, a significant proportion (approximately 50%) will have no further disease identified on re-excision specimens (109). Neither gross examination by palpation (150), intraoperative radiography (152) nor frozen section (153, 154) of specimens are reliable methods for real-time margin assessment of excised specimens. Recent studies utilizing radioguided occult lesion localization (ROLL) for non-palpable breast cancer did not demonstrate any difference in the proportion of negative margins achieved when compared with the gold standard wire-guided localization (155). Whilst intraoperative ultrasound guidance has shown a potential in reducing positive margins as compared with palpation-guided surgery, it may not be suitable for margin assessment where ductal carcinoma in situ (DCIS) and invasive lobular carcinoma (ILC) is involved (156). Additionally, it has also been reported that almost 50% of patients who undergo routine cavity wall shaving performed during BCS did not contain any evidence of residual disease within the shaved specimens (109). Finally, emerging techniques for intra-operative margin assessment such as radiofrequency spectroscopy (RFS) which provide the surgeon with feedback on the malignant potential of excised margins based on electromagnetic signatures (157) did not approach the accuracy of histopathological methods (158), and therefore have failed to lead to a reduction in the need for re-operative intervention in certain randomised clinical studies (159).

At present, the adequacy of local oncological treatment relies on the predictive value of histological assessment of the margins of excised tissues and yet the residual in situ disease burden i.e. cavity walls created following excision, is not known. Given that there is no evidence to prove that one particular margin width is more preferable to another, our theory is that it is not the arbitrary distance between the edges of the tumour that is most critical to outcome, but rather whether breast cancer cells have been inadvertently left in the patient. Rather than simply focusing on traditional histopathological assessment of the BCS specimens to guide the need for re-operation, arguably what is required are techniques that facilitate real-time assessment of the cavity bed and augment intraoperative decision making. The value of direct intraoperative assessment of the breast cavity wall has yet to be investigated arguably due to the lack of imaging technologies suitable for cavity deployment.
3.1.3 Potential role for pCLE

Probe-based confocal laser endomicroscopy (pCLE) is an emerging optical imaging tool that has the technical and physical properties to provide high resolution in vivo, in situ imaging of tissue morphology at cellular and subcellular level. The technique was initially developed to increase accessibility of the microscope for in vivo endoluminal tissue imaging and has since found promising clinical applications in surveillance of Barrett’s esophagus (35-37), assessment of indeterminate biliary strictures (160, 161) and pancreatic cysts (137). Utilizing fluorescent agents such as topical acriflavine hydrochloride and intravenous sodium fluorescein, morphological features of neoplastic and non-neoplastic tissues were shown to be readily visualized in real-time to the clinician during pCLE imaging. The ability of this flexible, hand-held and miniaturized endomicroscope to image neoplastic and non-neoplastic morphology in breast tissues as a potential real-time intraoperative imaging tool for in situ breast cavity wall assessment during BCS for breast cancer has yet to be systematically evaluated and in our opinion warrants further attention.

3.1.4 Aims and hypothesis

The hypothesis is that pCLE could potentially be used to distinguish breast cancer from normal breast tissues in real-time based on defining pCLE morphological features, utilizing acriflavine hydrochloride as a fluorescent nuclear staining agent (28). However, prior to assessing its potential to guide intraoperative decision-making, it is critical to first establish the morphological appearances of neoplastic and non-neoplastic breast tissues using histology as the gold standard for comparison. This study, performed on freshly excised breast tissues, demonstrates the feasibility of pCLE imaging towards intraoperative in situ margin assessment; and clarifies the ability of pathologists and surgeons to differentiate pCLE morphological images of neoplastic from non-neoplastic breast tissues using an in-house developed classification system.
3.2 Methods

3.2.1 Patients and tissue preparation

From December 2012 to January 2014, tissue samples were obtained after written consent from 50 patients undergoing wide local excision or mastectomy for histologically confirmed breast cancer at Imperial Breast Unit, Charing Cross Hospital (London, United Kingdom). Under an Imperial College London Institutional Review Board approved protocol (R12047a) for handling of human tissue, freshly excised cancer specimens were immediately transported to the pathology department where they were inked and underwent careful sectioning at 3- to 5-mm intervals by senior breast pathologists (S.S., R.R.). Each section was carefully examined by eye, palpation and specimen radiography where necessary, to locate the tumor. Thinly sliced samples measuring approximately 10 x 10 mm were retrieved from the center of tumor (n=50) and from macroscopically non-diseased appearing tissue away from the tumor margin (n=21) in mastectomy specimens (Figure 3.1). These samples were immersed immediately in acriflavine hydrochloride (AH) solution (0.01% in saline; Sigma Aldrich, UK) for 60 seconds and rinsed in normal saline solution.

3.2.2 Equipment and image acquisition

pCLE imaging of acriflavine-stained samples was performed using a pre-clinical probe-based confocal endomicroscopy system (Cellvizio, Mauna Kea Technologies, Paris, France) that consists of a 2.5 mm diameter flexible fibreoptic confocal miniprobe (Ultra Mini-O confocal miniprobe; Mauna Kea Technologies, Paris, France) connected to a laser scanning unit (LSU) that is connected to a standard personal computer for image data processing and display (Figure 3.2). The description of the pCLE system used in this study is detailed in Chapter 2 of this thesis.
Figure 3.1 Experimental workflow. (A) Freshly excised human mastectomy specimen. (B) Specimen carefully ‘breadsliced’ at 3-5 millimeter intervals. (C) Tissue samples obtained from the section containing macroscopically visible disease (red box) and from adjacent normal tissues (blue box) at least 30 mm away from the diseased site. Specimens were immersed separately in AH 0.01% solutions for 60 seconds.
Figure 3.2 Image acquisition of tissue cut-outs. (A) pCLE system; (B) Handheld pCLE miniprobe. The 488-nm excitation laser transmitted onto the tissue surface through a bundle of more than 10 000 optical fibers. Tissue samples imaged en face (insert). (C) An example of pCLE image mosaic created in the direction of probe movement.
3.2.3 Histopathology

After completion of imaging, each sample was placed in individually labeled cassettes, fixed in formalin and underwent routine histopathological processing. All samples were horizontally sectioned and evaluated by histopathological means to confirm the presence or absence of tumor based on formal appraisal of the haematoxylin and eosin (H&E) slides and the final tumor histological classification was assigned in accordance to the Cellular Classification of Breast Cancer of National Cancer Institute.

3.2.4 Correlation of image mosaics

All pCLE image mosaics depicting cellular morphology of tumor samples and non-diseased breast samples were stored digitally in specific folders into a prospectively maintained database. These were subsequently evaluated with two senior pathologists (S.S., R.R.). Each pathologist independently reviewed the morphological architecture depicted on all pCLE image mosaics and compared it with those seen on the corresponding histology slides. In particular, distinctive morphological features that were consistently observed across all pCLE images of each diagnostic entity (neoplastic vs non-neoplastic) were described in three distinctive categories: glandular, fibrous and adipose tissue components. During our analysis of glandular tissue component, careful attention was paid towards the size of ducts / acini, epithelial thickness and luminal cellularity. The evaluation of fibrous stromal component was focused on assessments pertaining to the relative degree of stromal cellularity and uniformity of stromal architecture. The adipose tissue component was focused on the recognition of individual fat cells and the degree of stromal cellularity interspersed between them. After the analysis of all pCLE image mosaics and respective histology slides were completed, a roundtable discussion between both pathologists and T.P.C., who had personal experience with the pCLE system, was carried out. The observations noted on each of these categories on pCLE image mosaics thought to be most consistent throughout our extensive analyses were discussed. All pCLE morphological features agreed by both pathologists were included as criteria in the new pCLE classification under the pre-
defined categories. All disagreements on the criteria for pCLE morphological features were resolved by discussion. No third party reviewer was required.”

3.2.5 Image interpretation assessment

To assess the feasibility of differentiating neoplastic from non-neoplastic tissues, nine pathologists and eight surgeons who were not involved in pCLE image acquisition of samples and had no previous training in pCLE image interpretation were recruited. Each participant underwent an in-house developed pattern recognition training session consisting of a 15-minute video tutorial during which representative pCLE image mosaics and its corresponding histology images were shown and pCLE features characterizing neoplastic and non-neoplastic breast morphology using our classification system were explained.

Subsequently, all participants were tested on a set of 50 de-identified pCLE image mosaics of histologically confirmed breast samples (25 neoplastic, 25 non-neoplastic) while blinded to the corresponding histology results. All pCLE images used in the training session were excluded from the test set. Each participant was asked to make a diagnosis of either neoplastic or non-neoplastic tissue. Neoplastic tissues included ductal carcinoma in situ and invasive carcinoma, whereas non-neoplastic tissues included normal breast tissues with no pathological changes. After diagnosing each pCLE image, the correct histopathological diagnosis was revealed to the participant.

3.2.6 Statistical analysis

The statistical analysis was performed using the statistical software package SPSS 21.0 (SPSS, Chicago, USA). The sensitivity, specificity, positive predictive value (PPV), negative predictive values (NPV), and accuracy of pCLE image interpretation were calculated, using the histology as the reference standard of diagnosis. The inter-observer agreement was expressed as the percentage of full agreement among the participants and by an overall kappa statistic with 95% confidence interval (CI). The interpretation of kappa values was done according to Landis and Koch (162).
3.3 Results

3.3.1 Acriflavine hydrochloride dose

Prior to the start of this study, a preliminary assessment was carried out on three tissue cut-outs obtained from neoplastic breast tissues. All three cut-outs were stained at AH concentrations of 0.01%, 0.05% and 0.08%, respectively. The latter two had excessive fluorescence retained despite repeated washouts with PBS solutions (Figure 3.3) and the underlying morphological architecture could not be discerned on pCLE images. Based on these, all tissue cut-outs were stained using 0.01% AH solutions.

Figure 3.3 pCLE images of AH-stained breast tissues at (A) 0.01%; (B) 0.05%; and (C) 0.08% concentrations.

3.3.2 pCLE image mosaics

The mean duration of pCLE imaging for each sample was 3 minutes (range, 1–6 minutes). A total of 350 pCLE image mosaics (mean 5 per sample; range 2 to 10) were carefully compared with histology features from seventy-one samples. With slow and controlled hand-held movements of the pCLE miniprobe, it was possible to generate high quality mosaics to expand the field of view by up to eight times (approximately 2 mm) to create more representative and interpretable pCLE images. The final
histopathology diagnosis from these samples showed 38 invasive carcinoma, 12 DCIS and 21 non-neoplastic tissues. No interference with H&E staining was noted in AH-stained samples.

3.3.3 Correlation of pCLE image mosaics with histology (non-neoplastic)

The nucleus of individual cells on pCLE image mosaics was visualized as fluorescent white dots. The glandular, fibrous and adipose tissues of normal breast tissues were discernible as shown in Figure 3.4
Figure 3.3 Each image depicts the morphological appearance on pCLE image (left panel) and the histology image of the corresponding sample (right panel). (A) A well-defined breast lobule with an aggregate of acini. Each acinar depicts a typical target-like appearance with dark-colored central lumen surrounded by a thin layer of homogenously lined fluorescent dots (nuclei of epithelium). (B) Fibrous connective tissue consisting of numerous fine, grey and linear strands of collagen fibers on a relatively acellular background. (C) Fibrous connective tissue with a mildly cellular stroma. Multiple small and discreet bright dots are seen scattered through the stroma. These represent the nuclei of individual fibroblasts. (D) Three adjacent dilated benign breast ducts are seen as larger-sized luminal structures with a thin layer of fluorescent dots. The lumen remained dark-coloured as it is acellular. (E) Elastic fibers visualized as wavy, bright-colored strands which can give an impression of a haphazard architecture. However, the background tissue remains scantily cellular as depicted by the sparsely populated nuclei. (F) Adipose tissue with numerous fat cells depicted as dark-colored, polygonal-shaped cells with thin well-defined bright borders. The nuclei of individual fat cells are visualized as distinct dots at its borders.

3.3.4 Correlation of pCLE image mosaics with histology (neoplastic)

In contrast, the breast ducts on ductal carcinoma in situ samples were visualized as markedly distended structures with hypercellular fluorescent dotted lumens. Invasive carcinoma had a distinctive appearance with increased cellularity and haphazard architecture characterized by hyperfluorescent infiltrating nests, sheets or files of tumor cells. In addition, pathognomic changes such as desmoplastic reaction and elastosis were noted in the adjacent stroma. Histopathological analysis of corresponding sample specimens from the same areas showed similar morphological architecture as highlighted in Figure 3.5.
Figure 3.4 Each image comprise of a pCLE image (left panel) and a histology image (right panel) of the corresponding sample. (A) Ductal carcinoma in situ: The epithelial lining of this duct appears markedly thickened and the luminal border of the epithelium is seen to be encroaching into the lumen. The entire duct appears markedly thickened and distended. (B) Invasive ductal carcinoma: Multiple irregularly organized clusters of fluorescent dots are seen infiltrating the stroma giving rise to a haphazard and disorganized appearance. Instead of the small and discreet bright dots of fibroblast nuclei, these clusters are larger in size and contain multiple fluorescent dots. A dense fibrous tissue response known as “desmoplastic reaction” is seen adjacent to these infiltrates producing a scirrhouos-like appearance. (C) Invasive ductal carcinoma: Broad sheet of densely packed tumour cells. (D) Ductal carcinoma in situ: The lumen of the duct is occupied by an island filled
with multiple fluorescent dots. (E) Invasive ductal carcinoma: another example of multiple clusters of tumour cells with stromal infiltration. (F) Invasive lobular carcinoma: Tumour cells are observed to be loosely dispersed in single linear rows. These are known as Indian file pattern, a pathognomonic sign of classical type invasive lobular carcinoma. (G) Metaplastic carcinoma with spindle-cell morphology: A relatively rare variant of invasive carcinoma with tumour cells seen here as adopting a spindle/comma shaped appearance. (H) Invasive tubular carcinoma: Tumour cells producing a ‘target cell’ appearance in the midst of a markedly hypercellular and elastotic background.

3.3.5 pCLE classification for breast morphology

Due to the technical differences of pCLE imaging compared with H&E histology (monochromatic images, smaller field of view), a unique pCLE classification system was developed which graduates neoplastic and non-neoplastic changes in three morphological entities (glandular, fibrous connective tissue and adipocytes) as shown in Figure 3.5. Utilizing our experience with pCLE imaging and breast histology, we postulated that the presence of residual deposits of carcinomatous foci on the edges of cavity walls could potentially be identified based on the presence of luminal or stromal hypercellularity, haphazard clustering of cells and loss of meaningful architecture.
<table>
<thead>
<tr>
<th>Non-neoplastic</th>
<th>Neoplastic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glandular component</strong> <em>(Figure 3.5a)</em></td>
<td><strong>Glandular component</strong> <em>(Figure 3.5c-d)</em></td>
</tr>
<tr>
<td>- Well-defined luminal structures</td>
<td>- Clusters of nucleated nests</td>
</tr>
<tr>
<td>- Little or no luminal celularity</td>
<td>- Increased luminal cellularity</td>
</tr>
<tr>
<td>- Minimal luminal distension</td>
<td>- Distended lumen</td>
</tr>
<tr>
<td>- Nucleated peripheries (fluorescent dotted)</td>
<td>- Peripheries contiguous with luminal cellularity</td>
</tr>
<tr>
<td><strong>Stromal component (fibrous)</strong> <em>(Figure 3.5b)</em></td>
<td><strong>Stromal component</strong> <em>(Figure 3.5d)</em></td>
</tr>
<tr>
<td>- Sparsely populated nuclei</td>
<td>- Abundance of nuclei</td>
</tr>
<tr>
<td>- Opaque-white ground substance</td>
<td>- Clusters of nucleated nests</td>
</tr>
<tr>
<td>- Sheets of parallel-lined fibres (collagen)</td>
<td>- Streaks of nucleated cords</td>
</tr>
<tr>
<td>- Interspersing wavy fibres (elastic)</td>
<td>- Opaque-white ground substance</td>
</tr>
<tr>
<td></td>
<td>- Haphazard appearance</td>
</tr>
<tr>
<td></td>
<td>- No uniform structures visualized</td>
</tr>
<tr>
<td><strong>Stromal component (adipose)</strong> <em>(Figure 3.5b)</em></td>
<td><strong>Stromal component (adipose)</strong> <em>(Figure 3.5d)</em></td>
</tr>
<tr>
<td>- Dark-coloured polygonal shaped cells</td>
<td>- Dark-coloured polygonal shaped cells</td>
</tr>
<tr>
<td>- Thin fluorescent borders</td>
<td>- Thin fluorescent borders</td>
</tr>
<tr>
<td>- Scantily populated nuclei at its borders</td>
<td>- Abundance of nuclei encroaching its borders</td>
</tr>
</tbody>
</table>

**Figure 3.5** New pCLE classification for neoplastic and non-neoplastic breast morphology with adjoining magnified views of image mosaics for visual comparison
### 3.3.6 pCLE image interpretation assessment

A total of 850 pCLE image interpretations were made by nine pathologists and eight surgeons (Table 1). When neoplastic pCLE morphological architecture was defined according to the combined changes to the glandular and stromal connective tissues as outlined in our classification, carcinomatous changes on pCLE image mosaics were predicted by pathologists with a sensitivity, specificity, PPV, NPV and accuracy of 96% (range, 88–100%), 92% (range, 84–100%), 90% (range, 69–98%), 100% (range, 71–100%) and 94% (range, 90–100%), respectively. Surgeons achieved a mean sensitivity of 97% (range 92-100%), a specificity of 86% (range, 68-96%), a PPV of 88% (range, 76–96%), an NPV of 97% (range, 92-100%) and accuracy of 92% (range 84-98%) (Table 3.1). The mean inter-observer agreement for pathologists was ’almost perfect’, κ=0.82 (95%CI, 0.79-0.85); and ‘substantial’ for surgeons, κ=0.74 (95%CI, 0.70-0.78).

<table>
<thead>
<tr>
<th>Histology diagnosis</th>
<th>Pathologists</th>
<th></th>
<th>Surgeons</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neoplastic</td>
<td>Non-neoplastic</td>
<td>Total</td>
<td>Neoplastic</td>
</tr>
<tr>
<td>Neoplastic</td>
<td>217</td>
<td>8</td>
<td>225</td>
<td>195</td>
</tr>
<tr>
<td>Non-neoplastic</td>
<td>17</td>
<td>208</td>
<td>225</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>234</td>
<td>216</td>
<td>223</td>
<td>177</td>
</tr>
</tbody>
</table>

Table 3.1. Correlation between pCLE image interpretation and gold standard histology.

The overall and participant-specific accuracy for pCLE images 1 to 10, 11-20, 21-30, 31-40 and 41-50 are shown in Table 3.2. The accuracy for pathologists was 94% (range 80-100%) for the first 10 pCLE images, followed by 96% (range 80-100%), 97% (90-100%), 97% (range 80-100%) and 89% (80-100%) in their respective image groups. Surgeons achieved accuracy rates of 86% (range 79-100%), 93% (80-100%), 96% (range 80-100%), 95% (90-100%) and 90% (70-100%) in the similar corresponding groups. When defining an acceptable accuracy rate of 90%, there were only 5 participants (63%) with an acceptable accuracy for the first 10 pCLE images, 6 participants (75%) for
the second 10, 7 participants (88%) for the third 10, 8 participants (100%) for the fourth and six participants (75%) for the final group.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Pathologists</th>
<th>pCLE images</th>
<th>1-10</th>
<th>11-20</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/10</td>
<td>8/10</td>
<td>10/10</td>
<td>8/10</td>
<td>9/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>9/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9/10</td>
<td>9/10</td>
<td>9/10</td>
<td>10/10</td>
<td>8/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9/10</td>
<td>10/10</td>
<td>10/10</td>
<td>9/10</td>
<td>9/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10/10</td>
<td>9/10</td>
<td>9/10</td>
<td>10/10</td>
<td>9/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>8/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>8/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>8/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>9/10</td>
<td>10/10</td>
<td>9/10</td>
<td>10/10</td>
<td>10/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Overall accuracy: 85/90 (94%) 86/90 (96%) 87/90 (97%) 87/90 (97%) 80/90 (89%)

No. (%) neoplastic: 3/10 (30%) 5/10 (50%) 5/10 (50%) 6/10 (60%) 6/10 (60%)

<table>
<thead>
<tr>
<th>Participant</th>
<th>Surgeons</th>
<th>pCLE images</th>
<th>1-10</th>
<th>11-20</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7/10</td>
<td>8/10</td>
<td>8/10</td>
<td>9/10</td>
<td>10/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9/10</td>
<td>8/10</td>
<td>10/10</td>
<td>10/10</td>
<td>9/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8/10</td>
<td>9/10</td>
<td>10/10</td>
<td>9/10</td>
<td>10/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>9/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8/10</td>
<td>10/10</td>
<td>9/10</td>
<td>10/10</td>
<td>8/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10/10</td>
<td>9/10</td>
<td>10/10</td>
<td>9/10</td>
<td>9/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>9/10</td>
<td>9/10</td>
<td>10/10</td>
<td>9/10</td>
<td>7/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Overall accuracy: 69/80 (86%) 73/80 (93%) 77/80 (96%) 76/80 (95%) 72/80 (90%)

No. (%) neoplastic: 3/10 (30%) 5/10 (50%) 5/10 (50%) 6/10 (60%) 6/10 (60%)

**Table 3.2.** Accuracy in pCLE images 1 to 10, 11 to 20, 21 to 30, 31 to 40, and 41 to 50 for each observer and overall.
3.4 Discussion

pCLE is an emerging imaging tool that enables real-time high resolution in situ subsurface imaging of tissue morphology. Whilst this technique has found promising endoscopic applications (29, 35, 90, 163), the ability to discriminate neoplastic from non-neoplastic breast morphology towards intraoperative in situ applications has yet to be systematically evaluated. This study is understood to be the first to report on the use of pCLE to facilitate prediction of breast cancer pathology on freshly excised specimens. By utilizing topical AH as a fluorescent agent and directly correlating pCLE images with the corresponding histology slides, common morphological architecture of glandular, fibrous and adipose tissue of normal human breast; and morphological changes that occur in the presence of carcinoma are readily identifiable on pCLE image mosaics.

3.4.1 Understanding pCLE breast morphology

Consistent with previous literature, AH crosses the cell membrane and displays a strong specificity for labeling acidic constituents (28, 29, 164, 165). It predominantly stains the nuclei of cells and has also been shown to demonstrate some staining specificity for collagen and elastin. Whilst AH is by definition a non-specific exogenous fluorescent agent, this study has shown that its relative affinity to these intra-cellular and tissue constituents has enabled sufficient delineation of breast morphological architecture to be made. For example, the presence of well-defined, individual fluorescent dots surrounding hollow dark lumens allows glandular structures to be easily distinguished from dark-coloured adipocytes, which have homogenously fluorescent and thin hexagonal borders. Similarly, the linear and branching strands of collagen and elastic fibres are readily identified as structural constituents of normal fibrous connective tissue.

By understanding common morphological changes that occur in carcinomatous disease using conventional histology, we were able to deduce the appearances of changes on respective pCLE image mosaics. In DCIS, the lumen of glandular structures are seen to be populated with hyperfluorescent
dots and form aggregates of islands, solid nests or thickened encroaching epithelium within a well-defined, distended glandular structure. Invasive diseases share common morphological patterns comprising architectures that appear markedly hypercellular and haphazard. The lack of any obvious principal organization is caused by the arbitrary passage of tumor cells through the stroma and these often evoke a dense fibrous tissue response which gives rise to a distinctive scirrhous-like morphological appearance on pCLE images. These stromal infiltrations exhibit a variety of morphological patterns on pCLE image mosaics i.e., nests, cords, clusters, solid sheet or linear files of hyperfluorescent tumor cells. The latter is commonly seen in invasive lobular carcinoma as its tumor cells are often found loosely dispersed in the stroma. Whilst these features are readily identified, it is important to note that distinguishing specific histological types of invasive disease such as invasive lobular from invasive ductal carcinoma on pCLE image mosaics alone is not possible as both frequently have overlapping morphological characteristics and thus require additional stains (e.g. E-Cadherin) on routine histology to establish definitive diagnosis. Furthermore, the importance of distinguishing between these two entities intra-operatively might be called into question given that the identification on cavity walls of either of these malignant pathologies mandates further excision.

3.4.2 pCLE breast classification

Whilst the new pCLE classification provided a structured and uniformed approach in pCLE image interpretation, it is important to note that considerable amount of work is still required to refine the objectivity of the diagnostic criteria. For example, we have yet to establish the predictive value of neoplasia for the presence or absence of specific morphological features outlined in each of the tissue components described in the classification. Consequently, one could argue that it is essentially a loosely defined and subjective classification, and that its effectiveness is highly operator dependent.

Having said that, most pCLE classifications on other clinical applications (GI tract, lung, urinary tract and biliary tree) (27, 37, 39, 51) were developed through consensus decision-making of expert pCLE users and a relative degree of subjectivity was deliberately allowed to avoid the pitfalls of the
inordinately objective approach. For example, the presence of goblet cells visualised on pCLE imaging of the large bowel is one of the features described for normal mucosa (37). However, its absence does not necessarily confer presence of neoplasia. Similarly, the absence of round-shaped and well-defined crypts do not necessarily indicate presence of adenomatous changes because hyperplastic changes can also produce slit-like crypt appearances (37). The relative subjectivity conferred by these classifications has yielded high predictive values and accuracies when it is used in clinical practice. Based on these observations, our proposed breast pCLE classification might have provided much of the basic criteria needed for interpretation but further efforts to evaluate the impact of refining the criteria should be carried out given the novelty of the intended clinical application.

3.4.2 pCLE image interpretation – a surgical armamentarium?

The clinical usefulness of morphological architecture visualized on pCLE image mosaics is largely dependent on the ability of its user i.e., the operating surgeon, to interpret these images in real-time during surgery. Previous studies in the gastrointestinal tract demonstrated that the prediction of neoplasia and non-neoplastic lesions based on pCLE imaging has a relatively high diagnostic accuracy (28, 35, 36) compared with histological analysis and clinicians were able to learn to recognize pCLE morphological features with a short learning curve (143). In this study, we have demonstrated that pCLE images of breast morphology can be learned rapidly by both groups of histopathologists and surgeons with a short training session that consists of offline exposure to a range of pCLE images and their corresponding histology slides. Both groups of clinicians were able to distinguish neoplastic from non-neoplastic pCLE breast morphology with an overall accuracy that exceeds 90% and of the 50 pCLE image mosaics interpreted, the proportion of pCLE images correctly interpreted was noted to be stable throughout five consecutive image cohorts.

During image interpretation, we observed that surgeons tend to err on the side of caution when encountered with equivocal non-neoplastic pCLE images and opted for a diagnosis of neoplasia. This may in part be due to the inherent anxiety of inadvertently leaving neoplastic tissues behind, a
common clinical dilemma faced by surgeons. Unsurprisingly, this is reflected in the slightly lower specificity and PPV attained by surgeons. Alternatively, the lack of familiarity with assessment of microscopic images may be contributory but we envisage that increasing experience and tailored training may improve accuracy. Whilst intra-operative assessment of margins using conventional frozen section or touch imprint cytology has been described (166-169), these approaches add on average 20-30 minutes to the operating time, lengthen time under anaesthesia and reduce operating theatre productivity (158). Indeed, the pressure on pathology services has grown steadily over the years and arguably reached a stage where these methods are arguably no longer practical. In our opinion, it is imperative that the breast surgeon be able to use and interpret pCLE images reliably without having to depend on the pathologist to do so in the operating theatre.

It is important to note that the inter-observer reproducibilities of conventional histopathological systems of breast cancer classification have historically yielded lower values [5]. In our feasibility study, we have shown that both surgeons and pathologists were able to attain high accuracies (>90%) and excellent interobserver agreements during pCLE image interpretation assessments. Whilst these results are highly promising, it must be stressed that image interpretation assessments were carried out based on high quality static pCLE image mosaics that clearly depicted a specific tissue entity. Additionally, these pCLE assessments were carried out in calm and controlled examination format. The combination of high quality and unambiguous pCLE images, facilitated with a relatively stress-free environment, had unsurprisingly facilitated an overall superior performance from the participants in this study. This raised questions as to whether these results could be reproducible in the presence of time-constraint, movement artefacts and multi-tasking pressures in a busy operating theatre. Evidently, the impact of these factors must be systematically evaluated so that any reduction in the overall accuracies or interobserver agreements are subjected to rigorous evaluation to identify whether surgical ergonomics or pathological description (e.g. inconspicuous morphological features of pCLE pattern recognition that were insufficiently addressed), or both, could be the perpetuating factor. Given that breast pathology is uniquely complex even with conventional histology, the assessments of
various critical pathognomic features on a monochromic background with limited field of view warrants careful evaluation.

3.4.3 Going beyond the endoluminal tract

Previous studies using benchtop confocal reflectance (9) and rigid confocal fluorescence microscopes (170) have reported that histological architecture of breast core biopsies and excision specimens respectively could be visualized in real-time. Whilst these microscopes may have a role for imaging resection margins of excised specimens, in situ imaging of cavity walls during surgery warrants significant miniaturization to enable insertion through a small 3-4 cm skin incision and for deployment against the lateral walls of the breast cavity. The hand-held fiber-bundle pCLE probe used in this study is 2.5 mm in diameter, light and flexible throughout its length with the exception of the distal 10 mm rigid tip which aids stable abutment of probe against the tissue surface.

We postulate that it would be ergonomically possible to deploy and orientate the distal tip of the pCLE probe perpendicular to the surface of the cavity walls for localized assessments guided by the intraoperative findings and as such the benefits of miniaturization for initial endoscopically-driven applications could potentially be extrapolated to applications outwith the confines of the endoluminal tract.

3.4.4 Envisaged optical requirements

Whilst miniaturization is essential for physical deployment, the optical properties of pCLE need to be equally optimized to provide adequate resolution and magnification for accurate differentiation of neoplastic from non-neoplastic tissues to be performed. Currently, pCLE image mosaics are generated with a lateral resolution of 1.0 µm. Based on the results of this study, we have established that this provided sufficient clarity to visualize common glandular and stromal characteristics of both neoplastic and non-neoplastic morphology.
Unlike conventional histology where images are magnified by switching between low and high power objectives, pCLE image frames are obtained under high power magnification with a limited field of view (240 x 240 µm). This renders image mosaicing an integral step to increase the field of view and to ensure representative pCLE images are obtained. This warrants slow and minute movements of the pCLE probe tip against the tissue surface and could potentially be time-consuming if extensive mosaics are to be created. A low power magnification probe may provide a wider field of view and potentially reduce the reliance on mosaicing. However, this may trade-off the clarity required to distinguish normal breast lobules from DCIS where small, well-defined epithelial-lined acini of a normal lobule could potentially be mistaken as constituents of a hypercellular lumen of DCIS on pCLE image mosaics. Similarly, adequate magnification is essential to facilitate differentiation of loosely dispersed tumor cells from non-diseased fibroblast nuclei.

Future work to ascertain the appropriate balance between magnification and field of view is required in order to optimize the economy of movement for intraoperative cavity assessment whilst minimizing the impact on pCLE image interpretability. Additionally, devising a pCLE probe movement pathway to facilitate time-optimized assessment of cavity walls may be required to prevent surgeons’ fatigue from excessive probe micromanipulations and this may require collaborative efforts from the integration of robotic technology with pCLE to provide a unique solution(95, 96).

3.4.5 Acute inflammatory response

It is equally important to note that much of the clinical value of an ergonomically optimized pCLE imaging platform depend on various biological factors that could potentially confound the cellular composition of tissue morphology. Evidently, pCLE image acquisition of excised tissues may depict the dynamic morphological changes seen in acute inflammatory response. Tissue trauma as a result of thermal injury from intraoperative diathermy is a stimulus for acute inflammatory response which leads to alterations in vascular caliber that causes an increase in blood flow, structural changes in the
microvasculature that permit plasma proteins and leukocytes to emigrate from the circulation and accumulate at the focus of injury (edges of the cavity walls).

The hallmark of the latter (cellular) phase is the aggregation of granulocytes, particular neutrophils on the margins of the cavity wall and this could potentially mimic neoplastic changes on pCLE image mosaics. However, this response takes place over a few hours as the leukocytes need to first undergo the process of margination and diapedesis before accumulating at the site of trauma, most typically during the first 6 to 24 hours. Given the estimated time to perform BCS is approximately 15-20 minutes from skin incision to tumor excision and that pCLE imaging of cavity walls is envisaged to be performed immediately after excision, it is unlikely that accumulation of leukocytes at the edge of the cavity wall will significantly influence assessment.

3.4.6 Benign breast conditions

It should be noted that there are benign breast conditions that could potentially mimic the morphological appearances of neoplastic diseases during pCLE imaging. For example, distinguishing benign conditions such as ductal hyperplasia of usual type and atypical ductal hyperplasia (ADH), from DCIS on pCLE image mosaics alone may be challenging as it may not provide adequate information on cytology, architecture and lesion size for accurate differentiation to be performed. Ductal hyperplasia of usual type does not require excision and findings of such could potentially lead to over exuberant resection should this be misinterpreted as DCIS. Potential false positives arising from this scenario require careful evaluation in the context of number needed to treat during larger intraoperative in situ studies.

Whilst findings of ADH on core biopsy warrants excision, there is insufficient evidence as to whether further excision is required when it is found on the margin of excised specimen. This is a controversial pathological diagnosis subject to large inter-observer variability and is beyond the scope of this paper. Nevertheless, it has been suggested that further excision of ADH at the surgical margin is considered
reasonable especially in the context resection for DCIS and therefore distinguishing between the two on pCLE images may not be critical. It remains to be stressed that the intended application of pCLE is not to substitute conventional histology, but rather as an intraoperative adjunct to facilitate detection of residual disease that are often occult and inconspicuously left behind at the time of surgery.

3.5 Conclusion

pCLE imaging of tissue morphology has emerged as a highly promising imaging tool in cancer research, offering real-time, non-invasive, in situ differentiation of tissues. Until now, pCLE technology has not been evaluated for use towards margin assessment of cavity walls in BCS. We have demonstrated the ability of pCLE to image morphology of neoplastic and non-neoplastic breast tissues and that clinicians are able to learn to differentiate these images. The findings presented here are promising and require validation in an in situ study with a sufficiently large cohort of patients.

We anticipate the clinical impact of pCLE to be the ability to identify the presence or confirm the absence of residual cancerous foci intraoperatively, thereby guiding operative decision-making during breast conserving surgery based on real-time in situ cavity scanning. In addition, our results provide a basis to develop an in-house pCLE system that addresses potential intraoperative challenges and to this end, further work is planned to improve the optical platform and mechatronic configurations to improve the field of view, image quality and consistency.
Chapter 4: Evaluation of Fluorescein-stained Breast Morphology on pCLE Images

4.1 Introduction

4.1.1 Overview

The findings from the previous chapter demonstrated that morphological features of neoplastic and non-neoplastic breast tissues were readily visualized and distinguished when acriflavine hydrochloride (AH) was used as the topical fluorescent agent. The nuclear-staining characteristics of AH have provided a distinct advantage by allowing definitive assessment of tissue cellularity to be carried out. Whilst this provided useful information pertaining to the presence of neoplasia, little is known about the morphological architecture of stromal component of neoplastic tissues. The presence of desmoplastic reaction, a fibrous tissue response to tumor cell infiltrations, was predicted based on the direction and organization of the thin streaks of tumor cells. Evidently, the morphological characteristics of adjacent tumor-induced fibrosis could not be clearly delineated. Whilst its affinity to fibrous connective tissues of non-neoplastic specimens was clearly elucidated, similar findings could not be deduced on that of neoplastic tissues despite the obvious presence of pathological fibrosis.

Intravenous fluorescein sodium (FS) is one of the few non-specific fluorescent agents that are compatible for use with pCLE imaging. Due to its partial binding to albumin, a small proportion of FS leaks into the surrounding parenchyma. Combined with its prominent vasculature, morphological architecture of FS-stained colonic crypts, gastric pits and gap junctions of gastrointestinal mucosa were readily delineated on pCLE images. Additionally, characteristic features of neoplastic changes of the corresponding tissues were distinguishable and agreed upon in several internationally developed classifications. However, its ability to stain fibrous connective tissues – a key component of the breast stroma that consists of fibroblasts, collagen fibres, elastic fibres and ground
substance, has yet to be elucidated. Previous studies utilizing a rigid confocal microscope probe (Optiscan©) have shown that fibrous tissues of the bowel mesentery were visualized and distinguishable from adipocytes (173). More recently, the fibrous component pancreatic cysts were also described on pCLE images as thin sheets of fluorescent fibres (68).

4.1.2 Hypothesis

Our hypothesis is that the use of intravenous FS could further improve delineation of the stromal component of neoplastic tissues. Equipped with that knowledge, differentiation between pathological fibrosis and normal fibrous tissues would be possible and that the use of AF as a topical fluorescent agent would serve as an adjunct to confirm the presence of neoplasia on pCLE images. By accentuating multiple tissue constituents, we hypothesis that higher quality and longer mosaics would be created given that pCLE’s ability to construct mosaics is partly dependent on the amount of morphological information captured in the preceding image frame.

4.1.3 Study design rationale

However prior to assessing its potential to guide intraoperative decision-making, it is important to first ascertain the morphological appearances of FS-stained tissues using conventional histology as the gold standard for comparison. To achieve this, the pCLE miniprobe needs to be deployed against the surface of a freshly sectioned tumor for image acquisition. For the purposes of this study, it is an onerous task to achieve as tumor deposits are often conspicuous. This may be ethically challenged as multiple tissue biopsies of the cavity walls might need to be performed to ascertain the pathologies of sites imaged. Whilst one could argue that the surgeon could potentially expose the tumor by diathermising through it in situ, this carries the risks of margin disruption and could potentially seed tumor cells in the cavity. In light of all these challenges, we decided that the best course of action would be for intravenous FS to be given shortly before exteriorization of the tumor is complete and pCLE image acquisition is carried out immediately as per protocol described in the previous chapter.
4.1.4 Study aims

The primary aim of this study is to establish the effect intravenous FS had on visualization of the stromal component of neoplastic (tumor) and non-neoplastic (non-tumor) breast tissues. The secondary aim is to ascertain whether provision of FS increased the lengths of pCLE image mosaics constructed as compared to that of AH-stained tissues.

4.2 Methods

4.2.1 Patients

In this prospective, cross-sectional, observational study, 10 patients who were at least 18 years of age and who underwent breast conserving surgery i.e., wide local excision (WLE) for biopsy confirmed breast cancer at a tertiary breast unit (Imperial Breast Unit, Charing Cross Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom) were recruited in this study. Each patient signed full informed consent prior to their surgery, which was approved by the institutional review board (West London REC 13/LO/1507). Patients who were pregnant, breast feeding, impaired renal function or had documented allergy to fluorescein were excluded from this study.

4.2.2 Intravenous fluorescein

All patients received between 1.5ml to 3.5ml of intravenous bolus of 10% fluorescein sodium (FS) (Martindale Pharmaceuticals, Romford, UK) in the operating theatre following induction of general anaesthesia. The timing of intravenous FS administration was scheduled to approximate the predetermined duration of intravascular dissemination prior to specimen exteriorization. The time of fluorescein injection, breast skin incision and specimen exteriorization was calculated to the nearest minute.
4.2.3 Specimen handling

Freshly excised WLE specimens were transported to the histopathology lab for immediate inspection by two experienced breast histopathologists (S.S., K.L.). All specimens had its margins inked and vertically sectioned to generate 3-5 mm thick slices. Specimen radiography was performed on all slices with the appropriate orientation preserved. Each slice was carefully examined to ascertain the location of the tumor while guided by findings on specimen radiography (Figure 4.1). Small cut-outs measuring 10 x 10 mm across were obtained each from the tumor and fibrofatty tissues distant from the tumor. Where cut-outs were judged not feasible to perform due to technical difficulties (small tumor size, impalpable or indistinct tumor) or risk of margin interference, the tumor was imaged in situ and digitally photographed thereafter to facilitate correlation with histology.

Figure 4.1 Freshly excised fluorescein-stained wide local excision (WLE) breast specimen with the surrounding margins inked. (A) Tumor site (shown between the forceps) identified during vertical sectioning. (B) Small cut-out containing the tumor for pCLE imaging. (C) Small WLE specimen with tumor approximating the margins. The tumor was imaged in situ as cut-outs might interfere with subsequent margin assessment.
4.2.4 Image acquisition and equipment

All cut-outs were imaged using a 488-nm wavelength laser probe-based confocal laser endomicroscopy (pCLE) system (Cellvizio, Mauna Kea Technologies, Paris, France) with a 2.5mm diameter, hand-held, confocal miniprobe (UltraMiniO, Mauna Kea Technologies, Paris, France) deployed perpendicular to the surface of the tumor. The equipment specifications are similar to those described in Chapter 2 of this thesis. The first set of image acquisition sessions were performed on the FS-stained tumor and fibrofatty cut-outs for up to five minutes each. These cut-outs were then subjected to further staining with 0.01% AH solutions for approximately 60 seconds and gently rinsed with phosphate buffer solution (PBS) to remove the excess fluorescence. A second set of pCLE imaging sessions were carried out on the previously imaged tumor and fibrofatty sites to facilitate comparison of FS-stained cut-outs with that of FS and AH-stained.

4.2.5 Correlation with histology

Each specimen cut-out was placed in individually labelled cassettes and returned for routine histopathology processing. Post-hoc evaluation of all pCLE images were carried out with the assistance of two experienced breast histopathologists (S.S., R.R.) using the corresponding histology slides as the gold standard for comparison. Both histopathologists had previous experience on interpretation of AH-stained pCLE images described in the previous chapter. The key features on FS-stained pCLE images thought to be predictive of morphological constituents of conventional histology were reviewed and agreed upon by consensus.

4.2.6 Evaluation of pCLE mosaic length

To evaluate the effects of FS and AH staining on pCLE mosaic construction, the top three longest combined FS and AH-stained mosaics from each patient in this study were retrieved. A randomised, single blinded selection from 10 patients with invasive carcinoma from the AH-stained tissue database used in the previous chapter was carried out and the top three longest AH-stained mosaics from each
patient were retrieved for comparison. The longest mosaic was defined as the longest length extending along the longitudinal axis of the image mosaic (Figure 4.2).

**Figure 4.2** Length of image mosaic was calculated along the centre-point in the longitudinal axis as depicted by the yellow dotted lines.

### 4.2.7 Statistical analysis

The inter-observer agreement was defined as the percentage of full agreement among the surgeons and by an overall kappa statistic with 95% confidence interval (CI). The interpretation of kappa values was performed according to Landis and Koch classification. Continuous variables were presented as mean and standard deviation (SD). Comparison of pCLE mosaic lengths between both groups of staining were performed using two-sample t-test. The statistical analysis was performed using the SPSS 22.0 software (SPSS, Chicago, USA).
4.3 Results

4.3.1 Baseline characteristics

All patients were female with a median age of 57 years (range, 48 to 84 years). None of the patients experienced any adverse reaction to FS, with the exception of transient yellow discoloration of the urine, which resolved within 24 hours after the operation. The median times between FS injection and breast skin incision and exteriorization of WLE specimens were 3 minutes (range, 2 to 25 minutes) and 18 minutes (range, 11 to 73 minutes), respectively. The median dose of FS injection in absolute volume was 1.5 ml (range, 1.0ml to 2.5ml). When this was calculated according to volume per kilogram, the median dose was 0.02 ml/kg (range 0.01-0.04 ml/kg). (Table 4.1)

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, sex</td>
<td>48, F</td>
<td>51, F</td>
<td>84, F</td>
<td>51, F</td>
<td>64, F</td>
<td>46, F</td>
<td>57, F</td>
<td>62, F</td>
<td>63, F</td>
<td>69, F</td>
</tr>
<tr>
<td>FS dose (ml)</td>
<td>2.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
<td>2.0</td>
<td>1.5</td>
<td>1.4</td>
<td>2.1</td>
<td>1.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78</td>
<td>77</td>
<td>44</td>
<td>81</td>
<td>67</td>
<td>69</td>
<td>72</td>
<td>71</td>
<td>46</td>
<td>78</td>
</tr>
<tr>
<td>FS dose (ml/kg)</td>
<td>0.03</td>
<td>0.02</td>
<td>0.04</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>eGFR</td>
<td>&gt;90</td>
<td>72</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>83</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Inj-Inc (min)</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>9</td>
<td>5</td>
<td>25</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Inj-Ext (min)</td>
<td>24</td>
<td>17</td>
<td>11</td>
<td>12</td>
<td>15</td>
<td>73</td>
<td>28</td>
<td>35</td>
<td>18</td>
<td>47</td>
</tr>
<tr>
<td>Pathology</td>
<td>IDC</td>
<td>IDC</td>
<td>IDC</td>
<td>IDC</td>
<td>IDC</td>
<td>IDC</td>
<td>DCIS</td>
<td>ILC</td>
<td>IDC</td>
<td>IDC</td>
</tr>
</tbody>
</table>

*FS*, Fluorescein sodium; *eGFR*, Estimated glomerular filtration rate; *Inj-Inc*, Injection to skin incision time; *Inj-Ext*, Injection to Exteriorization; *IDC*, Invasive ductal carcinoma; *ILC*, Invasive lobular carcinoma; *DCIS*, Ductal carcinoma in situ.

**Table 4.1** Baseline characteristics of patients undergoing BCS, intravenous FS dosage and circulation time.
4.3.2 Correlation with histology

Figures 4.3, 4.4, 4.5 and 4.6 summarize the findings of pCLE mosaics acquired from FS-stained tumor samples and following topical staining with AH whereas Figure 4.7 depicts the appearances fibrous connective tissues and adipocytes of normal breast tissues with the aforementioned stains.

**Figure 4.3** pCLE images and corresponding histology of invasive ductal carcinoma. (A) pCLE mosaic acquired from FS-stained tumor sites demonstrated a markedly hyperfluorescent appearance. These findings were in line with the presence of dense fibrous tissue response on the corresponding site on histology slides. Within these hyperfluorescent regions, multiple haphazardly arranged shades of white opaque patches were discernible giving rise to a heterogeneous texture. Additionally, numerous stellate-shaped dark spaces were seen interspersing this region and are likely to represent the ground substance of the stroma. (B) Invasive ductal carcinoma on histology with desmoplastic reaction visible in the stroma. (C) pCLE mosaics following additional staining with topical AH, the characteristic clusters and streaks of infiltrating tumor cells were seen embedded on these heterogeneous shades with the adjacent dark stellate-shaped spaces preserved.
Figure 4.4 pCLE images of infiltrating tumor cells from an invasive ductal carcinoma specimen. (A) pCLE mosaic of heterogeneously fluorescent regions depicting areas of tumor-induced fibrosis; (B) Histology image of infiltrating tumor cells evoking a desmoplastic reaction in the stroma; (C) pCLE mosaics of the corresponding area with bands and clusters of tumor cells (yellow arrows) surrounded by tumor-induced opaque white fibrosis.
Figure 4.5 pCLE images of infiltrating tumor cells admixed with adipocytes. (A) FS-stained specimens depicting a heterogeneously appearing opaque stroma (yellow arrows) suggesting the presence of pathological fibrosis around aggregates of dark-colored adipocytes. (B) Histology of the corresponding site containing invasive ductal carcinoma. (C) Following topical staining with AH, clusters of tumor cells (yellow arrows) were seen embedded on the opaque stroma.
Figure 4.6 pCLE images of ductal carcinoma in situ (DCIS) (A-B) Histology images of low grade DCIS. (C) The distended ducts of DCIS are clearly evident on FS-stained tissues. There are opaque material within the lumen of these ducts which might represent the presence of comedonecrosis. (D) Hypercellular epithelium encroaching the lumen (yellow arrows) were discernible. The presence of nuclei on the edges of these dark oval shaped structures confirms the presence of glandular structures and not that of adipocytes.
**Figure 4.7** pCLE images of the stromal component of normal breast tissues. (A-E) Following topical AH staining of FS-stained tissues, the borders of adipocytes are well delineated with nuclei visible on its edges. The fibrous tissues here appear relatively homogenously stained (yellow arrows) as compared to that of tumor-induced fibrosis. The haphazard and whirl-like background seen on tumor stroma is absent. (F) pCLE mosaic of adipocytes on FS-stained tissues prior to AH application.

The aforementioned morphological features seen on FS-stained tissues were readily visualized in nine patients who had their WLE specimens exteriorized between 11 to 47 minutes post-injection. One patient who had her specimen removed at 73 minutes post-injection had minimal fluorescence retained on both tumor and non-tumor sites. The dose of intravenous FS did not appear to have an effect on the overall tissue morphology.

### 4.3.3 Length of pCLE mosaics

The mean length (± SD) of pCLE image mosaics for combined FS- and AH-stained neoplastic tissues was significantly longer than that of AS-stained (1.47 mm ± 0.47 vs 1.08 mm ± 0.35; p= 0.001).
4.4 Discussion

4.4.1 Pathological fibrosis

To the best of our knowledge, this is the first study to describe the use of intravenous FS to visualise breast tissue morphology using pCLE. When injected intravenously, approximately 75-80% of the dye binds to plasma proteins such as albumin (28). The remaining unbound dye either remained intravascularly or diffused across the vessel wall to highlight the surrounding stromal tissues (28). It has been reported that fibrous connective tissues are readily visualised as fluorescent sheets of thin parallel lines on pCLE. However, its morphological appearance on fibrotic tissues induced by infiltrating tumor cells has yet to be ascertained.

It is important to note that fibrous connective tissues and fibrosis are two distinct entities. The former consists of collagen fibres that are present physiologically in the stroma whereas the latter denotes the abnormal growth of fibrous tissues in response to a stimulus i.e. trauma or neoplasia (174). In invasive breast cancer, infiltrating tumor cells often evoke a dense fibrous tissue response in the surrounding stroma. This led to overproliferation of fibroblasts and increased formation of collagen fibres which served as a scaffold to facilitate further infiltration of tumor cells (174). Evidently, this chain of events resulted in fibrosis and thereby depicted on pCLE images as fluorescent regions with haphazardly arranged, amorphous-looking collagen fibres.

4.4.2 Potential pitfalls of image interpretation

However, several potential pitfalls were observed during pCLE image evaluations. Indeed our ability to distinguish them emanated from the fluorescence intensity created by collagen fibres. Evidently, these haphazardly arranged collagen fibres appear prominent when pathological fibrosis is present. Conversely, they appear less distinct in its absence, as depicted on pCLE images obtained from normal fibrous tissues. Several non-invasive tumours such low grade DCIS are relatively indolent and may not evoke fibrous tissue response (175). Consequently, the lack these stromal responses on pCLE
images does not equate to absence of tumorigenesis. Whilst the fluorescent regions appear relatively heterogeneous on invasive tumors, the same could not be extrapolated to that of non-invasive tumors. Clearly, the presence of residual disease cannot be ascertained purely on the basis of pathological fibrosis on pCLE images and that an additional staining method that allows assessment of tissue cellularity remains pivotal.

4.4.3 Envisaged benefits of FS-staining

This study confirmed that AH provided more critical information pertaining to visualization of neoplastic diseases as compared to FS staining. Nevertheless, the presence of FS-stained stroma did not perturb the clarity of nuclei highlighted by AH. The combination did not result in excess fluorescence retained and their respective tissue staining affinities appeared to complement each other. The morphological features depicted by AH-stained tumor cells in this study were consistent with that of the previous chapter. Despite the lack of pivotal information, the FS-stained background conferred a halo effect which was congruent with the direction and pattern of tumor cell infiltration.

Collectively, the wealth of morphological features depicted by both stains facilitated the creation of longer pCLE mosaics. Given that AH confers superficial staining of tissues, changes to the depth of imaging that occur as a result of tissue deformation from miniprobe movements would inevitably impede further expansion of mosaics. When FS is added to the background stroma, the stain uniformity it imparts to all tissue layers allow mosaics to be further expanded when AH-stained layers intermittently fail to coincide with the optical slice imaged at the respective depth.

As mentioned in the previous chapter, the creation of expansive mosaics is envisaged to provide a distinct advantage in breast cavity wall scanning applications. Evidently, large scale coverage of tissue surface area could potentially be carried out in a time efficient manner without having to recommence orchestration of pCLE miniprobe movements whenever the preceding mosaic was prematurely halted. Given that the aim is to rule out the presence of residual deposits of tumor cells, it is evident that a
thorough coverage is required to reliably ascertain the local oncological status. The construction of multiple short mosaics poses the risk of inadvertently missing out on tumor cells that exist in the gaps between adjoining mosaics. Furthermore, definitive analysis of mosaics warrants clear and complete representations of any features deemed suspicious to the surgeon – a task that is difficult to achieve from short mosaics where partial or obscured views were often acquired.

4.4.4 FS-staining in the algorithm of cavity scanning

The concurrent administration of intravenous FS and topical AH has yet to be used in patients. Given the potential advantages in pCLE mosaic orchestration and the magnitude of the clinical problem addressed, further validation of its role for pCLE in situ detection of residual disease is clinically warranted. The timing and duration of FS staining do not appear to be a rate-limiting factor as the staining characteristics it confer is projected in the stroma within five minutes and remained so up to an hour post-injection without the need for additional doses. The envisaged application of intravenous FS in relation to pCLE imaging and topical application of AH is summarised in Figure 4.8.

It is anticipated that intravenous FS is administered after WLE specimen is exteriorized and haemostasis is achieved. The latter is critical to minimize coating of tissue surface with FS-containing blood. Whilst a small amount of dried blood is negligible, excessive oozing could potentially impede visualization of tissue morphology due to overfluorescence. The cavity wall that corresponds to suspicious findings on specimen inspection / radiography is then targeted for topical application of AH and pCLE imaging. Our experience dictates that one must be cognizant of the potential non-uniformity in the level of staining conferred by topical fluorescent agents. Where FS-stained fluorescent regions were judged to be heterogeneous, further application of AH solutions might be warranted to fully ascertain its degree of cellularity.
4.4.5 Study limitations

There are several limitations to this study. Firstly, it is performed on freshly excised and smoothly sectioned tumor specimens with post-hoc image evaluations performed in a controlled environment. It therefore does not take into account the challenges associated with intraoperative probe deployment, FS-containing haemorrhage and tissue surface irregularity. Secondly, this is a feasibility study on
small sample sizes which did not include non-invasive breast carcinomas such as DCIS. To this end, efforts to include mastectomy specimens with these tumor types are currently underway.

4.4.6 Conclusion

This study has shown that the morphological architecture of neoplastic and non-neoplastic breast stromal tissues were readily visualised when FS was used as the fluorescent agent. The presence of pathological fibrosis and its associated fibroblasts depicted a markedly heterogeneous appearance that comprise of haphazardly arranged fluorescent fibres. When combined with topical AH, infiltrating tumor cells were seen embedded on these heterogeneous regions. The creation of expansive pCLE mosaics was attributable to the full thickness staining that FS conferred. Intravenous FS might have role in facilitating identification of residual deposits of tumor cells and its combination with topical AH warrants intraoperative in situ breast cavity evaluation.
Chapter 5: Evaluation of Acriflavine-stained Parathyroid and Non-parathyroid Morphology on pCLE Images

5.1 Introduction

5.1.1 Minimally invasive parathyroidectomy

Surgery is the only definitive cure of primary hyperparathyroidism. A four gland parathyroid exploration has been for several decades the gold standard surgical treatment (176). However advances in preoperative localization imaging modalities such as high resolution ultrasound (US), sestamibi scintigraphy (SS) and increasingly 4D CT have allowed surgeons to identify patients who are suitable for a more focused, minimally invasive approach (176). Positive preoperative localization tests in patients with primary hyperparathyroidism mean that these patients can be offered targeted surgical cure without the need for more extensive surgical dissection and the associated additional risks (including the damage of normal PG), increase in operative time and poorer cosmesis (176).

5.1.2 Challenges with inconclusive localization tests

However, both US and SS are imperfect with sensitivity rates of detecting parathyroid adenomas ranged between 51 to 96% and 34 to 100%, respectively (122, 123), and both their sensitivities decrease further in the presence of multiglandular disease (MGD) (124, 125). Negative localization necessitates a bilateral neck exploration. Intraoperative identification of parathyroid disease in such circumstances is more challenging as the likelihood of MGD with parathyroid hyperplasia and even ectopic gland location is much higher (177). The appearances of tiny superficial nodules on the surface of a multinodular thyroid gland, adjacent ‘brown’ fat and small lymph nodes can often mimic the
appearances of a small parathyroid adenoma or a suppressed normal parathyroid gland. These challenges are equally pertinent to bilateral neck exploration for tertiary (renal) hyperparathyroidism and evidently more conspicuous in reoperative parathyroidectomy where accurate preoperative localization and visual discrimination of parathyroid tissues in obscured dissection planes are onerous to achieve (178).

Whilst ioPTH provides confirmation of the removal of the solitary parathyroid adenoma (179), it is less reliable in predicting the presence of MGD. Initial studies have shown that the use of ioPTH in MIP for primary hyperparathyroidism improved cure rates in patients with MGD (180, 181). However, other studies have reported that despite concordant preoperative localization scans and an appropriate PTH drop, additional abnormal parathyroid glands were found on complete exploration in up to 15% of patients (182). False positive reduction in ioPTH levels of 55% and 75% were also reported in the presence of double adenomas (183) and multiglandular disease (184), respectively. Additionally, the delayed renal clearance of PTH levels render ioPTH unsuitable for use during surgery for secondary (renal) hyperparathyroidism (185).

These findings suggest that the surgical treatment of primary hyperparathyroidism with negative localization studies is still challenging and that combined with the limitations of ioPTH, differentiating the mildly enlarged parathyroid gland from its mimics intraoperatively remains challenging. Evidently, what is required are imaging adjuncts that facilitate real-time assessment of tissue differentiation and facilitate intraoperative decision making. In particular, the role of in situ, real-time, virtual evaluation of morphological features of parathyroid tissues, the ‘optical biopsy’, has yet to be investigated arguably due to the lack of imaging technologies suitable for in situ deployment.

5.1.3 Potential role for pCLE

As mentioned in the previous chapters, pCLE is an emerging optical imaging tool that has the technical and physical properties to provide high resolution in situ, in vivo imaging of tissue
morphology at cellular level. It was initially developed to increase accessibility of the microscope for in vivo endoluminal tissue imaging by utilizing an optical fibrebundle to deliver its light source onto the tissue surface. It has since found promising clinical applications in the assessment of indeterminate biliary strictures(186), surveillance of Barrett’s esophagus (37), lung (26) and pancreatic cysts(68).

Utilizing fluorescent agents such as topical acriflavine hydrochloride (AH) and intravenous sodium fluorescein, morphological features of neoplastic and non-neoplastic tissues were shown to be readily visualized in real-time during pCLE imaging (26, 28, 29, 37). The ability of this flexible, hand-held, probe-based endomicroscope to image parathyroid and non-parathyroid morphology as a potential real-time intraoperative imaging tool for tissue differentiation during parathyroidectomy for primary and secondary hyperparathyroidism has yet to be systematically evaluated.

5.1.4 Aims and hypothesis

The hypothesis is that pCLE could potentially be used to distinguish parathyroid from non-parathyroid tissues in real-time based on defining pCLE morphological features, utilizing AH as a fluorescent nuclear staining agent. However, prior to assessing its potential to guide intraoperative decision-making, it is critical to first establish the morphological appearances of parathyroid and non-parathyroid tissues using conventional histology as the gold standard for comparison. This study, performed on freshly excised tissues, demonstrates the feasibility of pCLE imaging towards intraoperative in situ tissue differentiation; and establishes the feasibility of pathologists and surgeons to differentiate pCLE morphological images of parathyroid from non-parathyroid tissues.

5.2 Methods

5.2.1 Patients and tissue preparation

From April 2013 to December 2013, tissue specimens were obtained after written consent from 35 patients undergoing parathyroidectomy (n=23) for primary and secondary hyperparathyroidism and
thyroidectomy (n=12) for multinodular goitre (MNG) and malignant solitary nodule(s) at Department of Endocrine and Thyroid Surgery, Hammersmith Hospital (London, United Kingdom). Under an Imperial College Institutional Review Board approved protocol (R12083) for handling of human tissue, freshly excised parathyroid, thyroid and adjacent fibrofatty specimens were immediately transported to the pathology department where they were carefully sectioned by an experienced endocrine pathologist (R.F.). Parathyroid specimens were bisected whereas thyroid specimens were sectioned at 3- to 5-mm intervals and thinly sliced samples measuring approximately 10 x 10 mm were retrieved from the macroscopically non-diseased (non-neoplastic) section of thyroid specimens and from adjacent fibrofatty tissues. Based on our previous experience where 0.01% AH provided the best staining for breast tissues, we decided to compare the effects of three AH concentrations (0.005%, 0.01% or 0.05%) on parathyroid tissue staining. Each sample was stained immediately in one of three aforementioned AH concentrations (Sigma Aldrich, UK) for 60 seconds followed by gentle rinsing with PBS solution.

5.2.2 Equipment and image acquisition

pCLE image acquisition of AH-stained samples was performed using a probe-based confocal endomicroscopy system (Cellvizio, Mauna Kea Technologies, Paris, France) that consists of a pCLE miniprobe (Ultra Mini-O confocal miniprobe; Mauna Kea Technologies, Paris, France) connected to a laser scanning unit that is connected to a personal computer for image data processing and display (Figure 5.1). The pCLE system and miniprobe used in this study is similar to those used in Chapters 3 and 4 of this thesis. Further descriptions of the system are detailed in Chapter 2.

5.2.3 Histopathology

After completion of imaging, each sample was placed in individually labeled cassettes, fixed in formalin and underwent routine histology processing. All samples were horizontally sectioned and evaluated by formal appraisal of the haematoxylin and eosin (H&E) as per local protocol.
Figure 5.1 (A) pCLE system set-up with an example of an image mosaic depicted on the display screen. (B) pCLE miniprobe emitting a 488-nm, blue light excitation wavelength laser at its distal tip. (C) The tip of the pCLE miniprobe is placed perpendicular to the cut-surface of a freshly excised parathyroid adenoma specimen.

5.2.4 Correlation of image mosaics

All pCLE images depicting cellular morphology of parathyroid, thyroid and fibrofatty samples were stored digitally in specific folders into a prospectively maintained database. These were subsequently evaluated with an endocrine pathologist (R.F.) using the corresponding H&E stained histology as the gold standard for comparison. Following discussion between the pathologist (R.F.) and T.P.C., who had experience with the pCLE system, morphological characteristics that were thought to be distinctive on pCLE images and predictive for histological results were noted.
5.2.5 Image interpretation assessment

To assess the feasibility of accurately identifying specific tissue entities and differentiating parathyroid from non-parathyroid tissues on pCLE images, four pathologists and four surgeons who were not involved during pCLE image acquisition of samples and who had no previous training in pCLE image interpretation were invited to participate in an image interpretation study. Each participant underwent an in-house developed pattern recognition training session that consists of a 10-minute video tutorial during which characteristic cellular architecture of hypercellular parathyroid, non-diseased thyroid and fibrofatty tissues on H&E slides were elucidated.

Subsequently, the interpretation ability of all participants were evaluated based on a set of 70 de-identified pCLE image (32 hypercellular parathyroid, 26 normal thyroid, 12 fibrofatty tissues) of histological confirmed tissue entities while blinded to its corresponding histology results. For each pCLE image, each participant was individually asked to identify the tissue entity it most likely represents and to rank the difficulty of image interpretation on a five-point Likert scale (1 = very easy; 2 = easy; 3 = average; 4 = difficult; 5 = very difficult).

5.2.6 Statistical analysis

The statistical analysis was performed using the SPSS 21.0 software (SPSS, Chicago, USA). The sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) and accuracy of pCLE image interpretation were calculated, using the histology as the reference standard of diagnosis. The inter-observer agreement was defined as the percentage of full agreement among the participants and by an overall kappa statistic with 95% confidence interval (CI). The inter-observer agreement was calculated for each group of participants, resulting in six different kappa values each, namely the agreement of observer 1 and 2, 1 and 3, 1 and 4, etc. The interpretation of kappa values was performed according to Landis and Koch classification (162).
Level of difficulty of image interpretation between tissue types were analyzed using a one-way analysis of variance (ANOVA) with tissue types as the independent variable and Likert scores as dependent variable. When significance at the p<0.05 level was found, Tukey’s post hoc comparison was performed to compare pairs of means within the ANOVA. Comparison of Likert scale scores of pathologists to surgeons for overall and tissue-specific image interpretations were performed using two-sample t-test.

5.3 Results

5.3.1 Baseline pCLE image mosaic characteristics

The mean duration of pCLE imaging for each sample was 2 minutes (range, 1–5 minutes). Preliminary analysis of samples obtained from the first four parathyroid specimens revealed that minimal discernible architecture were visualized at 0.005% whereas over-fluorescence of tissues were noted at 0.05% AH concentrations (Figure 5.2). Meaningful morphological features were readily visualized at 0.01% AH concentration, consistent with the findings from our breast experiments in Chapter 3. Consequently, further analysis of all remaining samples was performed at the latter concentration.

A total of 178 pCLE image mosaics depicting cellular morphology (mean 5 per sample; range 2 to 10) were carefully compared with the corresponding histopathology slides from 35 samples. Utilizing image mosaicing for real-time feedback on the distal tip positioning, the miniprobe was moved slowly and studiously to facilitate construction of high quality pCLE image mosaics up to four to six times the original field of view (approximately 1 mm). Evidently, this enabled image acquisition of a wider range of morphological architecture displayed on the tissue surface and consequently, the pCLE images obtained are readily interpretable and representative of its respective samples. No interference with H&E staining was noted on AH-stained samples.
Figure 5.2 pCLE image mosaics of parathyroid morphology stained at various AH concentrations. (A) Excessive fluorescence noted at 0.05% concentration; (B) Individual nuclei delineated when 0.01% was used; (C) Some nuclei remain unstained at 0.005% and construction of image mosaics were not possible.

5.3.2 Correlation of pCLE image mosaics with histopathology

Consistent with the findings from previous chapters, the nuclei of individual cells are seen as discreet fluorescent dots on pCLE images. Equipped with this knowledge, the morphological architecture of parathyroid adenomas and hyperplasia are readily discernible on pCLE images as markedly hyperfluorescent dotted tissues with parenchymal cells arranged in densely packed nests and microfollicles. Other common morphological constituents in parathyroid tissues such as fibrovascular septa and cystic changes are visualised. (Figures 5.3, 5.4, 5.5 and 5.6)
Figure 5.3 Comparison of morphological architecture of hypercellular parathyroid tissues seen on histology (left panel) and pCLE image (right panel) of the corresponding sample. (A & B) Hypercellular parathyroid parenchyma are seen here as numerous fluorescent dots organized in little nests and appear more densely packed in Image B. Fat cells are invariably absent in parathyroid adenoma and sparsely admixed within the parenchyma in parathyroid hyperplasia samples. (C) Microfollicular variant of parathyroid hyperplasia.
Figure 5.3 (cont.) Comparison of morphological architecture of hypercellular parathyroid tissues seen on histology (left panel) and pCLE image (right panel) of the corresponding sample. (D) Another example of microfollicular variant of parathyroid hyperplasia. (E & F) The parathyroid vascular network, represented by numerous fibrovascular septa are characterized here by interconnecting dark-coloured, hollow-looking, relatively acellular cord-like / slit-like spaces (yellow arrows) that traverse the densely packed parenchymal cells giving rise to a trabeculated appearance.
Figure 5.4 Comparison of cystic changes on hypercellular parathyroid tissues seen on histology (A) and pCLE images (B and C). Histology from a section on a large 20-mm parathyroid adenoma depicting multiple small cystic spaces interspersed with parenchymal cells. These are represented on pCLE images by multiple pockets of capacious, amorphous and acellular spaces.
Figure 5.5 Magnified views of pCLE mosaics of parathyroid microfollicular architecture interspersed with adipocytes.
Figure 5.6. Magnified views of pCLE mosaics of fibrovascular spaces within the parathyroid parenchyma. Nests of parenchymal cells seen between these spaces.

Similarly, the epithelial-lined follicles of thyroid tissues are easily discernible and are distinct from parathyroid tissues on pCLE images (Figures 5.7 and 5.8). These are distinguishable from adipose tissue which has an acellular thin rim of cytoplasm surrounding a central dark-coloured vacuole. Constituents of stromal tissues such as collagen and elastic fibers with fibroblast nuclei are discernible (Figure 5.7).
Figure 5.7 Comparison of non-parathyroid tissues on histology (left panel) and pCLE images (right panel). (A) Thyroid follicles are seen as dark-coloured, luminal structures, lined by a discreet layer of fluorescent dots depicting that of an epithelium. Several lumens are seen permeated with opaque grey-like substance which connotes the presence of colloid material. (B) A thyroid follicle with adjacent fibrous stroma.
Figure 5.7 (cont.) Comparison of non-parathyroid tissues on histology (left panel) and pCLE images (right panel). (C) Adipose tissues populated by dark-colored, polygonal-shaped cells. (D) Fibrous tissues that populates the thyroid stroma.
Figure 5.8 pCLE images of thyroid tissues depicting follicles of various sizes and shapes consistent with that of a multinodular goitre specimen.
5.3.3 Accuracy of pCLE image interpretations

A total of 560 pCLE image interpretations were carried out by four pathologists and four surgeons. The accuracy of pCLE tissue type identification were predicted by pathologists and surgeons with a mean accuracy of 94% (range 93-94%) and 93% (range 91-94%), respectively. The most common misidentification on pCLE images by pathologists occurred between parathyroid and thyroid tissues, which accounted for 15 out of 17 (88%) errors. Surgeons on the other hand committed a wider range of errors involving all three tissue types. Accounting for 52% (11 out of 21) errors, surgeons most commonly misidentified thyroid for parathyroid tissues (11 out of 13 errors) (Table 5.1). The mean inter-observer agreement for tissue type identification among pathologists was ‘almost perfect’, κ=0.86 (95%CI, 0.82-0.90); and ‘substantial’ among surgeons, κ=0.80 (95%CI, 0.75-0.85).

<table>
<thead>
<tr>
<th>Pathologists</th>
<th>pCLE diagnosis</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology diagnosis</td>
<td></td>
<td>Thyroid</td>
<td>Parathyroid</td>
<td>Adipose / Fibrofatty</td>
<td>Total</td>
</tr>
<tr>
<td>Thyroid, n (%)</td>
<td>97 (93)</td>
<td>6 (6.0)</td>
<td>1 (1.0)</td>
<td></td>
<td>104</td>
</tr>
<tr>
<td>Parathyroid, n (%)</td>
<td>9 (7.0)</td>
<td>119 (93)</td>
<td>0 (0)</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>Adipose / Fibrofatty, n (%)</td>
<td>0 (0)</td>
<td>1 (2.0)</td>
<td>47 (98)</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>126</td>
<td>48</td>
<td></td>
<td>280</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Surgeons</th>
<th>pCLE diagnosis</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology diagnosis</td>
<td></td>
<td>Thyroid</td>
<td>Parathyroid</td>
<td>Adipose / Fibrofatty</td>
<td>Total</td>
</tr>
<tr>
<td>Thyroid, n (%)</td>
<td>91 (87)</td>
<td>11 (11)</td>
<td>2 (2.0)</td>
<td></td>
<td>104</td>
</tr>
<tr>
<td>Parathyroid, n (%)</td>
<td>4 (3.0)</td>
<td>124 (97)</td>
<td>0 (0)</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>Adipose / Fibrofatty, n (%)</td>
<td>1 (2.0)</td>
<td>3 (6.0)</td>
<td>44 (92)</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>138</td>
<td>46</td>
<td></td>
<td>280</td>
</tr>
</tbody>
</table>

*Table 5.1* Correlation between tissue type identification on pCLE images and histology
To illustrate the potential difficulties in interpreting pCLE images, several examples of commonly made errors are shown in Figure 5.9.

**Figure 5.9** Examples of errors encountered during pCLE image interpretation using typical thyroid follicles for comparison (A). B, Thyroid follicles mistakenly identified as adipocytes due to the visibly sparse epithelial nuclei. C, Parathyroid microfollicles misinterpreted as that of the thyroid. D, Adipocytes with a central cluster of
fibroblast nuclei misidentified as parathyroid tissues. E, Cystic spaces of parathyroid tissues thought to represent thyroid follicles. F, Multinodular goitre with follicles of varying sizes misinterpreted as parathyroid tissues due to adjacent degenerative changes.

When thyroid and adipose/fibrofatty tissues were grouped together as non-parathyroid tissues, parathyroid tissues on pCLE images were predicted by pathologists with mean sensitivity, specificity, PPV and NPV of 93% (range, 88%-100%), 95% (range, 89%-100%), 94% (range, 89%-100%) and 94% (range, 90%-100%), respectively.

Surgeons achieved a mean sensitivity of 97% (range 91%-100%), a specificity of 91% (range, 84%-100%), a PPV of 90% (range, 84%-100%) and an NPV of 97% (range, 93%-100%) (Table 5.2).

<table>
<thead>
<tr>
<th>Histology diagnosis</th>
<th>pCLE diagnosis</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pathologists</td>
<td>Surgeons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parathyroid</td>
<td>Non-parathyroid</td>
<td>Total</td>
<td>Parathyroid</td>
<td>Non-parathyroid</td>
<td>Total</td>
</tr>
<tr>
<td>Parathyroid</td>
<td>119</td>
<td>9</td>
<td>128</td>
<td>124</td>
<td>4</td>
<td>128</td>
</tr>
<tr>
<td>Non-parathyroid</td>
<td>7</td>
<td>145</td>
<td>152</td>
<td>14</td>
<td>138</td>
<td>152</td>
</tr>
<tr>
<td>Total</td>
<td>126</td>
<td>154</td>
<td>280</td>
<td>138</td>
<td>142</td>
<td>280</td>
</tr>
</tbody>
</table>

Table 5.2. Correlation between pCLE image interpretation and histology on parathyroid and non-parathyroid tissues

5.3.4 Level of difficulty of pCLE image interpretation

The overall mean level of difficulty score for surgeons was significantly lower than that of pathologists and remained significantly lower when categorised into correct and incorrect interpretations. The mean scores for both pathologists and surgeons were notably higher for incorrect interpretations. When grouped according to tissue-specific categories, the mean scores were
significantly higher for pathologists on parathyroid tissues as compared to surgeons (Table 5.3). The overall level of difficulty of image interpretation based on type of tissues assessed was significantly different and remained so when grouped according to pathologists and surgeons (Figure 5.10).

<table>
<thead>
<tr>
<th>Specialty</th>
<th>Overall (n=560)</th>
<th>Tissue-specific</th>
<th>Tissue-specific</th>
<th>Tissue-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n=560)</td>
<td>Correct (n=522)</td>
<td>Incorrect (n=38)</td>
<td>Thyroid (n=208)</td>
</tr>
<tr>
<td>Pathologists (n=4)</td>
<td>3.1 ± 1.4</td>
<td>3.0 ± 1.4</td>
<td>4.5 ± 0.8</td>
<td>2.9 ± 1.5</td>
</tr>
<tr>
<td>Surgeons (n=4)</td>
<td>2.6 ± 1.2</td>
<td>2.5 ± 1.2</td>
<td>3.8 ± 0.9</td>
<td>2.9 ± 1.2</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>0.016*</td>
<td>0.839</td>
</tr>
</tbody>
</table>

Table 5.3 Comparison of mean level of difficulty of assessments on pCLE image interpretation measured on a five-point Likert scale grouped according to tissue type and specialty. Data are presented as mean ± SD. * denotes p value of <0.05

Post hoc analysis demonstrated significant pair-wise differences between the mean scores and all tissue types for pathologists; and between thyroid and fibrofatty tissues for surgeons (Figure 5.10).
**5.4 Discussion**

This study is to our knowledge the first to report on the use of pCLE to obtain high-resolution, real-time images of parathyroid and non-parathyroid morphology on freshly excised specimens. By utilizing topical AH as a fluorescent agent and directly correlating with the corresponding histology slides, the morphological architecture of parathyroid and non-parathyroid parenchymal cells are readily visualized and distinguishable by both pathologists and surgeons with high accuracies.
5.4.1 Pattern recognition

The ability of pCLE to differentiate tissue morphologies emanated from the preferential binding of AH to cell nuclei and staining specificity for collagen and elastic fibers (27-29). Whilst it is commonly described as a non-specific fluorescent agent, it is evident that its relative affinity to these intra- and extracellular constituents has endowed the clinician with three pivotal tissue staining characteristics from which specific morphological features can be deduced.

- Firstly, its nuclei staining characteristics has enabled assessment of tissue cellularity to be made objectively. Whilst this readily differentiates parathyroid from non-parathyroid tissues, we envisage that a potential clinical application of pCLE would be to distinguish diseased from non-diseased parathyroid tissues in situ intraoperatively when equivocal glands are encountered. Evidently, the findings of sparsely populated fat cells or its absence in the presence of hypercellular parathyroid morphology on pCLE images should raise suspicion that the observed gland is diseased and thereby warrants excision.

- Secondly, the relatively anucleate structures such as fibrovascular spaces, follicular lumens, cystic spaces, central vacuole of fat cells provide a dark contrast to its neighboring nucleated constituents. The lack of fluorescence retention in these regions enables recognition of coherent patterns that facilitates differentiation of important structural entities e.g. uniformly lined thyroid follicle from irregularly shaped cystic spaces and finger-like projections of fibrovascular spaces.

- Lastly, its affinity to collagen and elastic fibers provide a homogenously hazy backdrop which indicates the presence of structural component in the adjacent stroma instead of vacuous spaces.

It should be noted that given the hypercellular nature of the tissues imaged, a lower concentration of AH (0.01%) was expectantly found to provide the best staining characteristics for tissue morphology visualization as compared those used in the aforementioned studies (0.05%).
5.4.2 Image interpretation

As mentioned in the previous chapters, it is clear that the clinical value of real-time pCLE image acquisition is determined by the ability of the operating surgeon to interpret these images accurately and efficiently. Previous pCLE studies have shown the morphological features of neoplastic and non-neoplastic changes obtained from gastrointestinal mucosal lesions could be predicted in real-time by the endoscopist with accuracies of over 90% (45, 47). Additionally, the learning curve for pCLE image interpretation was reported to be short following structured training sessions (47). These studies, however, used intravenous fluorescein as a contrast agent, and when coupled with the different tissue types analyzed, render objective comparison with our study difficult to perform.

Nevertheless, our study demonstrated that by utilizing a short and focused training session on basic histology, surgeons with no prior experience in pCLE image interpretation were able to acquire relevant pattern recognition skills to differentiate parathyroid from non-parathyroid tissues with overall accuracies and interobserver agreements that approximate to that of the pathologists. Interestingly, pathologists experienced more difficulties interpreting pCLE images of parathyroid tissues than surgeons and consequentially committed most of their image interpretation errors in this tissue cohort.

This could plausibly be explained by the fact that the vast experience pathologists possess could have led to over-interpretation of pCLE images and more importantly, that their performance may project a more realistic representation of the potential complexities and pitfalls that could occur when differentiating parathyroid from thyroid tissues.

Evidently, smaller sized follicular structures could well be that of the thyroid gland and similarly, the collapsed edges of the epithelial lining of parathyroid cystic spaces could be misidentified as the infolding of thyroid follicles, a common metabolic consequence of an overactive thyroid gland that has just been rendered euthyroid at the time of surgery (187). This undoubtedly warrants further
analysis on a wider morphological variant of parathyroid and thyroid tissues and its effect on image interpretation.

Nonetheless, unperturbed by the complexities, surgeons were able to acquire the basic skills to differentiate pCLE images of different tissue types. Furthermore, both pathologists and surgeons recorded a higher mean level of difficulty when errors were made which suggests that both groups demonstrated insight when faced with equivocal pCLE images.

5.4.3 Intraoperative deployment

Real-time assessment of morphological features of parathyroid tissues using bench-top confocal reflectance microscopes have been reported by White and colleagues (8). Similar to our findings, tissue cellularity and fat cells were readily observable in diseased and non-diseased parathyroid tissues, albeit having a lower resolution than the pCLE used in our study. However, the feasibility of image interpretation by surgeons was not systematically evaluated. Additionally, it is not miniaturized and therefore unsuitable for intraoperative deployment for in situ assessments.

Our evaluation of pCLE’s feasibility towards surgical applications could potentially represent an advent of bringing the microscope to the patient in the operating theatre and we anticipate that several physical characteristics it possesses make it suitable for intraoperative use. The current diameter of 2.5 mm at the miniprobe tip is essential for optimal surface area contact as most normal parathyroid glands measure 3 to 6 mm in the largest dimension. The flexible, 90 cm miniprobe length used in this study allows placement of the LSU adjacent to the operating table, convenient deployment of the miniprobe onto the operative field at the surgeon’s accord and devoid of cumbersome manoeuvres of repositioning when assessment of multiple tissue sites are required. With the exception of the distal 2cm tip, the flexibility it confers allows it to be bent to achieve perpendicular contact against the tissue surface without compromising image quality.
### 5.4.4 Potential intraoperative challenges

On the other hand neighboring respiratory movements could arguably impede the creation of high quality mosaics and thereby impact on the interpretability of the images generated. The technique of creating pCLE mosaics is akin to the notion of path dependency whereby accurate spatial localization of the subsequent image frame is highly dependent on the clarity and amount of morphological features imaged in the preceding frame. This undoubtedly warrants steady, controlled and purposeful probe tip movements whilst utilizing the concurrently generated pCLE mosaic as a real-time feedback tool to further orchestrate its expansion. However, given that image acquisitions are performed at high magnifications, these are expectantly susceptible to minimal movements which cause the images to be less distinct and potentially introduce artifacts. Whilst these are well described in endoluminal environments (37, 92), our experience in this study suggests that similar challenges are pertinent to the hand-held approach.

A potential strategy to address this challenge would be to reduce over-reliance on the need for excessive mosaicking by increasing the field of view of individual frames. These modifications warrant a systematic evaluation to establish the optimal trade-off between resolution, field of view and probe diameter. Whilst the size constraints pertaining to probe diameter might not constitute a critical requisite as in the case with endoscopic applications, any enlargement of probe diameter needs to take into account the clinical accessibility of deployment and optimization of adequate tissue surface contact.

Other possible approaches to circumvent the challenge with physiological movements could potentially necessitate the integration of robotic technologies (96) and hand-held force adaptive instruments (92, 93) to minimize inadvertent probe tip movements. Preliminary results from pre-clinical studies in endoscopic- and cavity-based tissue models have shown promising results (92, 93, 96) and we are currently exploring the feasibility of applying these technological adjuncts to an in-house developed pCLE system to parathyroid surgery.
5.4.5 Study limitations

There are several limitations to this study.

- First, it is performed on freshly excised specimens with post-hoc image interpretation assessments performed in a controlled environment and therefore does take into account the challenges associated with probe deployment and real-time decision-making in a time-constraint operating theatre environment. Whilst we do not envisage pCLE assessments to be time-consuming, a formal appraisal of its potential impact on the operative time needs to be assessed.

- Second, we did not differentiate diseased from non-diseased parathyroid tissues in this study due to ethical concerns of removing normal parathyroid glands when it is clinically not indicated and the associated risks of hypoparathyroidism. Additionally, it is difficult to harvest an incidental parathyroid gland from a thyroid specimen given our institution’s low rates of inadvertent parathyroidectomy (120).

- Third, lymphatic tissues such as lymph nodes are potential mimics of parathyroid glands and therefore warrant evaluation in prospective in situ studies.

5.4.6 Conclusion

This study has described a novel and innovative approach to visualization of morphological architecture of parathyroid and non-parathyroid tissues utilizing pCLE, an emerging imaging tool, to provide real-time, high magnification imaging at cellular resolution. The pivotal findings include excellent correlation of pCLE images with gold standard histology and feasibility of attainment of pattern recognition skills by surgeons.

We envisage that this could potentially be a useful adjunct to visual identification of parathyroid tissues without the removal of the tissue and to provide the surgeon recourse by performing multiple assessments when encountered with several equivocal tissues. Based on these promising findings, the
priorities for future research include systematic evaluation of in situ imaging acquisition of parathyroid glands and adjacent soft tissues; objective differentiation between diseased and non-diseased parathyroid glands; methodological quantification of the aforementioned intraoperative challenges; and assessment of optical trade-off requirements and mechatronically enhanced technological adjuncts as a potential solution to these challenges.
Chapter 6: Evaluation of Vascular Morphology of Fluorescein-Stained Parathyroid Glands on pCLE Images

6.1 Introduction

6.1.1. Postoperative hypoparathyroidism

Postoperative hypocalcemia is a well-recognized complication of total thyroidectomy. The incidence of transient hypocalcaemia after total thyroidectomy range from 24 to 37% whereas permanent hypocalcemia has been estimated at 0.3 to 8.3% (136, 188). The latter is a major concern as the consequences of chronic hypocalcaemia is often insidious, debilitating and could potentially lead to life threatening complications (188-190). Additionally, it is also a common cause of medico-legal litigation (191). In the majority of cases, postoperative hypocalcemia following total thyroidectomy is secondary to impairment of parathyroid function – a consequence of inadvertent removal, traumatic injury or devascularisation of parathyroid glands (PG) during dissection (188). It is widely advocated that if the devascularised PG was recognised intraoperatively, these glands should be autotransplanted to a neighbouring muscle, most commonly the sternocleidomastoid, to preserve parathyroid viability and function (192-194).

6.1.2. Challenges with PG viability assessment

Whilst the decision to autotransplant the excised gland or conspicuously transected vascular pedicle is relatively straight forward, the same cannot be iterated for those that are of uncertain viability such as those that are discoloured from traumatic injury. A study by Promberger et al showed that the function
of discoloured PGs are only transiently impaired and that full recovery returns shortly after surgery (135). It has also been suggested that autotransplantation should not be the first-line management of discoloured PG in the absence of other risk factors (135). Given that PG discolouration may indicate the presence of venous stasis or possibly haemorrhagic infarction, most surgeons would however choose to autotransplant these to maximise function preservation (192-195).

On the other hand, the absence of discolouration is an equally unreliable method of confirming PG viability. A study by Kuhel and Carew showed that devascularized or excised PGs did not appear discoloured at the time of surgery (134). The authors reported that when incisional biopsies were performed to ascertain its vascular supply, these PGs demonstrated no evidence of active capsular bleeding. Evidently, the reduction in blood flow or devascularisation has not inflicted obvious morphological changes that were discernible to the surgeon within the short time frame.

The lack of a reliable method to ascertain viability of PG remains a course for concern. The relevance of this limitation extends to all forms of neck surgery where the aim is to retain as much functional PG as possible. Despite meticulous dissection utilizing the well-described technique of capsular dissection, there remains a small proportion of PG who will inevitably lose their function through inadvertent removal or devascularisation.

In light of the prevailing nature of this problem, several groups have suggested that routine autotransplantation should be performed as a prophylactic measure to minimize the risk of postoperative hypoparathyroidism (193, 196). However, the removal of the undamaged gland appears to offer no reduction in the incidence of permanent hypoparathyroidism. Whilst autotransplantation is a well-established procedure, it harbours a small risk of graft failure (success rate range between 85 to 99%) and therefore a selective approach is usually preferred to minimize inflicting unnecessary injury to the unperturbed PG.

Despite the obvious limitations, routine decisions pertaining to selective autotransplantation still rely on the predictive value of visual assessment of PGs and yet the in situ vascular supply to these glands is still not known. Rather than simply focusing on the predictive values of operative procedures (e.g.
central neck dissections), thyroid conditions (Grave’s disease, recurrent goitre, thyroid carcinoma) and serum measurements (preoperative calcium and intraoperative PTH levels) to guide the need for postoperative supplementation, arguably what is required are techniques that facilitate real-time assessment of the viability status of PG and augment intraoperative decision making.

6.1.3 Vascular morphology on pCLE imaging

Utilizing intravenous fluorescein sodium (FS) as the contrast agent, vascular morphology of superficial vessels in the gastrointestinal tract mucosa, bladder mucosa and biliary epithelium were readily discernible during pCLE image acquisition (63, 172, 197, 198). In particular, the morphological characteristics displayed by the respective vascular architecture on pCLE images forms an essential component of tissue neoplasia assessment (28, 37). The presence of dilated, tortuous and elongated vessels visualized alongside distortion of crypt or gastric pit architecture are well recognised characteristics of neoplastic changes – all evidently visible on pCLE image acquisition (37).

However, most descriptions pertaining to vascular morphology focus on the static appearances of these vasculatures. In particular, little has been reported pertaining to visualization of vessel flow as potential indicators of tissue viability. In light of the aforementioned challenges in PG viability assessment, it would be prudent to investigate whether pCLE has a role in confirming viability of these glands intraoperatively.

6.1.4 Aims and hypothesis

The hypothesis is that pCLE could potentially be used to distinguish viable from non-viable PG in real-time based on defining dynamic morphological features.

However, prior to assessing its potential to guide intraoperative decision-making, it is critical to first establish the morphological appearances viable and non-viable PG utilizing carefully preserved and excised PG, respectively as the gold standard for comparison.
This pilot study aims to demonstrate the feasibility of pCLE imaging towards intraoperative in situ viability assessment; and clarifies the relationship between pCLE vasculature findings and post-operative PTH function.

6.2 Methods

6.2.1 Patients

The study protocol was approved by the institutional review board (West London REC 14/LO/0016) and all patients provided written, informed consent. Between March to July 2014, a total of 20 patients undergoing parathyroidectomy for primary hyperparathyroidism and thyroidectomy (total and completion) for benign and malignant thyroid diseases were prospectively recruited to the study from a tertiary endocrine surgery unit (Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom). Patients who were pregnant, breast feeding or had previous documented allergy to intravenous sodium fluorescein were excluded from this study.

6.2.2 Equipment

Image acquisition of vascular morphology of PG tissues was performed using a probe-based confocal endomicroscopy system (Cellvizio, Mauna Kea Technologies, Paris, France) that consists of a pCLE miniprobe (Coloflex, Mauna Kea Technologies, Paris, France) connected to a laser scanning unit (LSU) that is connected to a personal computer for image data processing and display. The system description for LSU was described in Chapter 2 of this thesis. However the pCLE miniprobe used in this study has slightly different optical properties. The optical slices of images were obtained at approximately 10 µm thickness. These were created in real-time at a depth of 90 µm below the tissue surface with a field of view of 240 x 240 µm and lateral resolution of 1 µm. Similar to previous studies, all images were scanned with a rate of 12 frames per second, hence demonstrating a real-time video on a display screen.
6.2.3 Intraoperative pCLE image acquisition

Two experienced endocrine surgeons (F.P., N.T.) with a combined volume of more than 400 endocrine cases per year performed all the operations and pCLE image acquisitions. All patients received between 1.5 to 3.0ml of intravenous bolus of 10% sodium fluorescein (Martindale Pharmaceuticals, Romford, UK) intraoperatively at least five minutes prior to commencement of pCLE imaging. Utilising a sterile-draped, hand-held mini-probe, pCLE image acquisition was obtained following perpendicular contact of the miniprobe tip with the exposed surface of PG. The stack system is positioned across the operating table in direct view of the surgeon so that the concurrently generated pCLE images were used as a real-time feedback tool to orchestrate the direction of miniprobe movement (Figure 6.1).

![Figure 6.1](image)

**Figure 6.1** pCLE system set-up in the operating theatre. (A) The display screen is placed within the vicinity of the operating surgeon. (B) The pCLE miniprobe is draped with a sterile transparent sheath that is reinforced with a layer of Tegaderm at its distal tip to ensure uniform abutment of the miniprobe tip with the PG surface during image acquisition. (C-D) In patients undergoing parathyroidectomy, further imaging is performed following excision or devascularisation of the PG.
6.2.4 pCLE imaging for parathyroidectomy

For patients undergoing parathyroidectomy, pCLE imaging was performed on the diseased PG with its vascular pedicle carefully preserved to ensure that its viability was maintained. In order to validate the vascular morphology visualized, further pCLE imaging was repeated immediately on the same site of the diseased PG following devascularisation or excision for gold standard comparison.

6.2.5 pCLE imaging for thyroidectomy

For patients undergoing total or completion thyroidectomy, every effort was made to isolate as many normal PG where it was operatively safe to do so. With its respective vascular pedicles carefully preserved, pCLE imaging was performed on normal PG towards the end of the operation, i.e. following excision of the diseased thyroid gland but prior to fascia closure. All normal PGs imaged were judged to be viable on the basis of its macroscopic appearance and preservation of its vascular supply. The decision to preserve PG in situ was made prior to the commencement of pCLE image acquisition and this remained unchanged following completion of imaging. All pCLE videos were stored digitally in specific folders into a prospectively maintained database for post-hoc analysis and comparison with post-operative calcium and parathyroid hormone levels (PTH).

6.2.6 Assessment of pCLE vascular morphology

Preliminary evaluations of pCLE videos between the surgeons (F.P., N.T.) and T.P.C., who had clinical experience with the pCLE system, were carried out during and after image acquisitions where morphological characteristics that were thought to be distinctive of vascular flow were noted.

To independently assess the validity of our observations, a structured assessment was designed to systematically evaluate recognition of vascular morphology on each pCLE video based on the following components: video stability, vessel recognition, vessel flow, flow rate and level of confidence (Figure 6.2). Assessments of video stability were performed according to an in-house developed classification (Figure 6.3).
To evaluate the interobserver agreements and accuracies of individual component assessments, six surgeons who had previously reviewed pCLE videos of non-parathyroid vascular architecture from the published literature were invited to independently assess each pCLE video obtained from PGs using the aforementioned structured approach while blinded to patient details and macroscopic appearance of its respective PGs.

Video assessments were carried out in two distinct cohorts, namely Cohort 1 which consists of video clips retrieved from pre-excision and post-excision/devascularized pCLE imaging of parathyroid adenomas, followed by Cohort 2 that was composed of those acquired from preserved normal PGs.
6.2.7 Comparison with post-operative PTH and calcium levels

The findings on vascular morphology assessment for pCLE videos in Cohort 2 were compared with post-operative serum calcium and PTH levels taken on the night after surgery and the following morning at 2100 and 0600 hours, respectively. Post-thyroidectomy hypocalcaemia and hypoparathyroidism was defined as corrected serum calcium levels of < 2.10 mmol/l (reference range 2.15-2.60 mmol/l) and PTH levels of < 1.0 pmol/l (reference range 1.1-6.8 pmol/l) on the first
postoperative day 1 (POD1), respectively. The lowest corrected calcium and PTH levels on POD1 were used to define POD1 hypocalcaemia and hypoparathyroidism.

6.2.8 Statistical analysis

The statistical analysis was performed using the SPSS 22.0 software (SPSS, Chicago, USA). The inter-observer agreement was defined as the percentage of full agreement among the participants and by an overall kappa statistic with 95% confidence interval (CI). The inter-observer agreement was calculated for each surgeon, namely the agreement of observer 1 and 2, 1 and 3, 1 and 4, etc. The interpretation of kappa values was performed according to Landis and Koch classification.

For Cohort 1, the sensitivity, specificity and accuracy of pCLE prediction of viability were calculated using the pre-excision PGs (adenomas) as the gold standard reference for viability. Comparison of Likert scale ratings were performed using two-sample t-test and the association between the ratings were determined using Spearman rank correlation.

For Cohort 2, comparison between pCLE video evaluations and post-operative PTH and calcium levels were presented using simple percentage agreement between six observers.

6.3 Results

6.3.1 Baseline characteristics

Twelve patients underwent total thyroidectomy for primary malignancy (n=6) and multinodular goitre (MNG) (n=6), seven patients had parathyroidectomy for primary hyperparathyroidism and one had completion thyroidectomy for residual malignant disease. For parathyroidectomy patients, a total of 22 pCLE video clips were extracted from 14 image acquisition sessions that were acquired from seven diseased PG. Of the 14 sessions, seven were performed prior to excision and the remaining was repeated on the same PG following devascularization.
All PGs imaged were confirmed histologically as hypercellular parathyroid tissues. For total and completion thyroidectomy patients, thirty-three pCLE video clips were retrieved from 23 PG. All patients completed pCLE image acquisition with no adverse events reported. All pCLE videos were presented as 10-20 second anonymized short clips.

6.3.2 Vascular morphology description

Consistent with published literature on pCLE vascular morphology from non-parathyroid tissues, blood vessels on PGs were readily visualized on pCLE videos as well-delineated tubular structures containing individual erythrocytes, each represented by dark-colored, discrete, mobile particles against a bright backdrop of fluorescein-containing plasma.

A wide spectrum of vessel architecture were discernible, ranging from networks of capillaries with vessel diameter as small as 10-µm, to larger sized single or branching vessels up to 100-µm in diameter. Within these vessels, the presence of blood flow was distinctively recognized on viable PG as unidirectional thrusts of erythrocytes whereas complete cessation of flow was noted on devascularised PG.

Variations in the flow dynamics were readily discernible with brisk vessel flow characterized by high velocity, steady unidirectional stream of erythrocytes whereas sluggish flow was depicted as lower velocity momentary spurts interspersed with brief moments of cessation of flow or retrograde movements. Adjacent stromal tissue constituents such as adipocytes and fibrous connective tissues were also visualized (Fig. 6.4)
Figure 6.4 pCLE images of PG vascular morphology with the vascular pedicle preserved. The respective vasculatures are denoted by the yellow arrows: (A) Interconnecting network of capillaries; (B) Large vessel with individual erythrocytes (red arrow); (C) Single vessel in between aggregates of intraparenchymal fat cells; (D) Branching and tortuous looking vessels; (E) Overlapping vessels; (H) Branching vessels with adjacent adipocytes. Asterisk (*) denotes fat cells.
Figure 6.5 pCLE images of PG vascular morphology following devascularisation or excision. (A) Network of capillaries with empty and contracted lumens (dark coloured); (B) Empty branching vessels interspersing the parenchymal adipocytes; (C) Parallel empty vessels; (D) FS containing network of capillaries with no erythrocytes visible within the lumen; (E) Single vessel with FS within. No erythrocytes seen; (F) Single vessel containing FS with static erythrocytes visible (red arrow).
Figure 6.6 Magnified views of pCLE image mosaics of blood vessels and individual erythrocytes
Figure 6.7 An example of pCLE image mosaic of the vast networks of capillaries on preserved PG
Figure 6.8 pCLE image mosaic depicting PG branching vessels and fibrous connective tissue (bottom half)
6.3.3 Vascular morphology evaluation

A total of 132 structured evaluations of pCLE videos were performed in Cohort 1. Of these, 98 (74%) assessments had blood vessels judged to be sufficiently visualized. Of the 34 assessments where vessels were not visualized, 28 (82%) were imaged from non-viable PG and six (18%) were from viable PG. The mean stability of all 132 evaluations was 3.6 \( \pm \) 1.0 (mean \( \pm \) SD). The overall interobserver agreement for vessel recognition was moderate (\( \kappa = 0.52; \) 95 CI% 0.41-0.63). Based on the remaining 98 pCLE videos, further assessments on vessel flow and flow rate demonstrated excellent (\( \kappa = 0.95; \) 95% CI 0.90-1.0) and strong (\( \kappa = 0.62; \) 95 CI% 0.49-0.75) interobserver agreement, respectively (Table 6.1).

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th></th>
<th>Group B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kappa (( \kappa ))</td>
<td>95% CI</td>
<td>Agreement</td>
<td>Kappa (( \kappa ))</td>
</tr>
<tr>
<td>Vessel recognition</td>
<td>0.52</td>
<td>0.41-0.63</td>
<td>Moderate</td>
<td>0.38</td>
</tr>
<tr>
<td>Vessel flow</td>
<td>0.95</td>
<td>0.90-1.0</td>
<td>Excellent</td>
<td>0.86</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.62</td>
<td>0.49-0.75</td>
<td>Strong</td>
<td>0.68</td>
</tr>
<tr>
<td>Mean ( \pm ) SD</td>
<td>3.6 ( \pm ) 1.0</td>
<td></td>
<td>3.4 ( \pm ) 1.0</td>
<td></td>
</tr>
<tr>
<td>Spearman's correlation</td>
<td>( r_s = 0.49, p &lt; 0.001 )</td>
<td></td>
<td>( r_s = 0.51, p &lt; 0.001 )</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1 Interobserver agreements on components of vascularity assessment, and correlation between pCLE video stability and level of confidence

When viable PG on pCLE videos were objectively defined as the presence of brisk or sluggish flow, the presence of non-viable PG were predicted with an overall sensitivity, specificity, PPV, NPV and accuracy of 92% (range 78-100%), 97% (range 90-100%), 95% (range 83-100%), 95% (range 83-100%) and 95% (range 85-100%), respectively (Table 6.2). The overall mean for level of confidence of assessments was 3.9 \( \pm \) 0.9 (mean \( \pm \) SD) and this was moderately correlated with that of video stability (3.6 \( \pm \) 1.0 (mean \( \pm \) 1.0) (\( r_s = 0.49, p < 0.001 \)).
Table 6.2 Correlation between pCLE vessel flow assessments and intraoperative evaluation on viability status of parathyroid glands from Cohort 1.

For Cohort 2, a total of 198 pCLE evaluations were carried out, of which 29 (15%) had vessels deemed insufficiently visualized. The mean stability of all 169 evaluations were $3.4 \pm 1.0$ (mean ± SD) and the interobserver agreement on vessel recognition was fair ($\kappa=0.38$; 95 CI% 0.27-0.49). The remaining 169 assessments demonstrated comparable interobserver agreement to that of Cohort 1 on vessel flow ($\kappa=0.86$; 95 CI% 0.78-0.94) and flow rate ($\kappa=0.68$; 95 CI% 0.60-0.76). Similarly, there was moderate correlation between the level of confidence of assessments ($4.0 \pm 0.8$ (mean ± SD)) and video stability ($3.4 \pm 1.0$ (mean ± SD)) ($r_s=0.51, p<0.001$).

6.3.4 Comparison with post-operative PTH function

Post-operative bloods showed that 10 out of 12 patients (83%) who had presence of blood flow on at least one of the PG identified had normal PTH levels with normocalcaemia or POD1 hypocalcaemia which resolved within four weeks (Table 6.3). Of the three patients (23%) who developed post-operative permanent hypoparathyroidism, two had both presence and absence of vessel flow discerned on pCLE videos obtained from the one and only PG visualised, whereas the remaining patient had complete cessation of vessel flow on videos obtained from two PG. All respective video assessments of vessel flow in these three patients had full percentage agreement by the assessors. When distinguishing vessel flow rate on pCLE videos, the proportion of assessments with full percentage agreement for sluggish flow was significantly higher than that of brisk flow (92% vs 42%, p=0.01).
<table>
<thead>
<tr>
<th>Pt No.</th>
<th>Video No.</th>
<th>PG imaged</th>
<th>Primary disease</th>
<th>Op</th>
<th>POD1 ↓CCal</th>
<th>POD1 ↓PTH</th>
<th>Vessel flow</th>
<th>Agreement (%)</th>
<th>Flow rate</th>
<th>Agreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Right sup</td>
<td>MNG</td>
<td>TT* Yes</td>
<td>Absent</td>
<td>Absent</td>
<td>2/2 (100%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Right inf</td>
<td>MNG</td>
<td>TT No</td>
<td>Present</td>
<td>Absent</td>
<td>5/5 (100%)</td>
<td>Brisk</td>
<td>1/2 (50%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>Right sup</td>
<td>Papillary CA</td>
<td>TT Yes</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Brisk</td>
<td>4/6 (67%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>Left sup</td>
<td>Papillary CA</td>
<td>CT Yes</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Brisk</td>
<td>6/6 (100%)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>Left sup</td>
<td>Papillary CA</td>
<td>TT Yes</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Brisk</td>
<td>6/6 (100%)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>Right sup</td>
<td>Large MNG</td>
<td>TT No</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Brisk</td>
<td>6/6 (100%)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>Right sup</td>
<td>Papillary CA</td>
<td>TT CND</td>
<td>Present</td>
<td>Absent</td>
<td>3/3 (100%)</td>
<td>Sluggish</td>
<td>3/3 (100%)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>Right sup</td>
<td>Papillary CA</td>
<td>TT LND</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Brisk</td>
<td>6/6 (100%)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>Right sup</td>
<td>Follicular CA</td>
<td>TT Yes</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Sluggish</td>
<td>4/6 (67%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>Right sup</td>
<td>Large MNG</td>
<td>TT No</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Sluggish</td>
<td>6/6 (100%)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>24</td>
<td>Left inf</td>
<td>Follicular CA</td>
<td>TT CND</td>
<td>Present</td>
<td>Absent</td>
<td>5/6 (83%)</td>
<td>Sluggish</td>
<td>5/6 (100%)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>28</td>
<td>Left inf</td>
<td>MNG</td>
<td>TT No</td>
<td>Present</td>
<td>Absent</td>
<td>5/6 (83%)</td>
<td>Sluggish</td>
<td>5/6 (100%)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>30</td>
<td>Right sup</td>
<td>Large MNG</td>
<td>TT No</td>
<td>Present</td>
<td>Absent</td>
<td>2/2 (100%)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>Right inf</td>
<td>MNG</td>
<td>TT No</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Brisk</td>
<td>5/6 (83%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>Right inf</td>
<td>MNG</td>
<td>TT No</td>
<td>Present</td>
<td>Absent</td>
<td>6/6 (100%)</td>
<td>Brisk</td>
<td>3/6 (50%)</td>
<td></td>
</tr>
</tbody>
</table>

*PG, Parathyroid gland; Op, Operation; POD1 ↓CCal: Post-operative Day 1 hypocalcaemia; POD1 ↓PTH: Post-operative Day 1 hypoparathyroidism; MNG, Multinodular goitre; CA, Carcinoma; TT, total thyroidectomy; CT, Completion thyroidectomy; CND, Central neck dissection; LND, Lateral neck dissection

* Had autotransplantation of one PG and inadvertent excision of another PG which was not autotransplanted.

**Table 6.3** Post-operative corrected calcium and parathyroid hormone levels compared to vessel flow and flow rate assessments on pCLE videos from Cohort 2.
Figure 6.9 pCLE images depicting presence and absence of vascular flow on PG from three patients (A & B) Empty vessels seen on both PGs from Patient 1; (C) Single vessel with vascular flow from Patient 4; (D) Parallel empty vessels on the same PG from Patient 4; (E) Large and small vessels with vessel stasis from Patient 7; (F) Network of capillaries with sluggish flow on the same PG from Patient 7.
6.4 Discussion

6.4.1 Overview

Utilising intravenous fluorescein as the contrast agent, vascular morphology on PG was readily
visualized during pCLE image acquisition and variations in vessel flow rates were distinguishable by
surgeons. When excised or devascularized PG was used as the gold standard comparison for non-
viability, characteristic features of viable and non-viable vascular morphology were deducible with
high accuracies and interobserver agreement.

Preliminary validation on preserved PG during total and completion thyroidectomies revealed that the
presence of blood flow on PG did not confirm viability of preserved PG. Instead, those with empty or
static vessels detected on the sole PG identified during high risk operations demonstrated post-
operative hypoparathyroidism. These findings demonstrate the importance of detecting empty / static
vessels on pCLE images and highlight the need for a robust method to improve vessel recognition
through strategic modifications of the current pCLE system and better pattern recognition training.

6.4.2 Visualization of vascular morphology

Consistent with previous pCLE studies on endoluminal applications (27, 28), vascular morphology of
PG were distinguishable within minutes of intravenous fluorescein administration and remained
discernible up to 90 minutes post-injection. Our ability to visualise PG vasculature is emanated by the
relative difference in fluorescence intensity between the vessels and its surrounding parenchyma.
Intravenous fluorescein binds strongly to serum albumin, thereby retaining most of the substance in
the vessels. A fraction of the unbound molecules diffuses pass the vessel wall and enter the interstitial
spaces, highlighting the extracellular matrix of parenchymal tissues (25). Evidently, this attributes the
prominent cell walls of interspersing adipocytes, parallel-lined collage fibres and relatively dark
particulate appearance of cellular components of the blood. Elucidation of fluorescein-stained chief
and oxyphil cells is not feasible at present in light of its non-nuclear staining properties and
additionally, distinguishing these cells on conventional histology rely largely on the relative differences on its cytoplasmic color shades – an endeavour that is not possible on monochromatic pCLE images.

Nevertheless, our primary focus is on vessel visualization which plausibly explains our observations that lower fluorescein doses of 1.5 to 2.0 ml provided sufficient contrast as compared to those used for gastrointestinal tract applications (~ 2.5 to 5.0ml) which often mandates a diffuse and uniformly stained parenchyma in addition to vascular morphology for diagnostic interpretation. Additionally, this study also confirmed that the relatively thin PG capsule has negligible light scattering effect as pCLE imaging from the subcapsular and superficial parenchymal vasculature was feasibly achieved.

6.4.3 Vessel recognition

Whilst approximately 20% of all structured evaluations demonstrated absence of vessels, the findings from our control arm in Cohort 1 showed that more than 80% of these were from non-viable tissues. Unsurprisingly, the absence of organised columns of particulate movements renders vessel discernment conspicuously difficult given that these tubular structures could adopt several possible appearances.

- Firstly, the lack of propulsive forces meant that pooling of erythrocytes at pre-capillary sphincter regions could result in network of capillaries appearing as featureless white bands akin to that of adipocyte cell walls.

- Secondly, the lack of uniform dispersement of fluorescein renders these tubular structures a dark hollow appearance. Although these are potentially recognisable on larger sized arterioles where luminal patency is maintained, vasculature such as post-capillary venules would expectantly collapse when it is empty, rendering elucidation of its thread-like appearance extremely challenging.
Lastly, despite the wealth of descriptions on pCLE vasculature, there is paucity of published information pertaining to that of empty or static vessels. Consequently, our vessel recognition ability during assessments might represent the early stages of the learning curve and could potentially improve with increasing experience.

6.4.4 Vessel flow and flow rate

Nevertheless upon definitive recognition of these vessels, assessment of flow could be determined with excellent interobserver agreements. Utilizing the control group in Cohort 1, the findings of complete cessation of vessel flow on devascularized PG confirmed that our observations were indeed that of the vasculature and not movement artefacts. Once vessel flow has been confirmed, further scrutiny into flow rates pertaining to brisk and sluggish vessel flow could be ascertained with strong interobserver agreements.

Whilst this may appear relatively straightforward on still pCLE videos depicting lone vasculatures, the task becomes less conspicuous when presented with videos of vessels with various sizes. Evidently, the brisk flow observed on larger sized vessels could translate into intermittent movements within the capillary beds depicting that of sluggish flow. Indeed these physiological variations need to be factored into future assessments where flow rates are localized objectively to their respective vessel type, i.e. large, medium or small vasculature. The clinical question that needs to be answered here is does the presence of concurrent sluggish and static flow on larger and smaller sized vessels; respectively indicate impending non-viability of the PG.

6.4.5 Comparison with post-operative PTH function

Perhaps the most intriguing observation in this study is that the presence of vessel flow alone did not confirm viability of preserved PG. Instead those with concurrent empty or static vessels adjudicated
with full agreement by assessors on sole PG identified during high risk operations were found to develop post-operative hypoparathyroidism. The marked variations in vessel flow within the same PG confirms that the adequacy its blood supply are indistinguishable macroscopically and profoundly capricious. Additionally, our observations could suggest that there might be watershed regions within the PG indicating its susceptibility to ischemia. In the presence of vessel flow, one would expect collaterals to develop around the non-vascularised regions to salvage the PG function. However, the lack of PTH levels post-operatively indicated that this did not occur sufficiently to preserve viability.

It is important to note that conclusions on the resultant PTH levels could not be definitively attributed to the specific PG assessed in this study. Instead the findings of absent flow on those assessed might suggest that devascularisation to the other PG had occurred as a result of inadvertent traumatic injury or excision that were not appreciated during surgery.

6.4.6 Future work

In our quest of ascertaining PG viability status, the findings of this study suggest that recognition of vessels with no vascular flow is of paramount importance. Evidently, this requires an extensive and detailed appraisal of the PG vasculature, one that is onerously difficult to achieve with the current small field of view that the miniprobe confers.

Adaptive modifications on both the optical configurations and physical characteristics of the current pCLE system are warranted to facilitate image acquisition of a wider surface area. Importantly, this require systematic evaluation of optimal trade-offs between pCLE image resolution and miniprobe size for intraoperative deployability. Additionally, integration of the miniprobe with robotic-actuated or mechatronically-enhanced devices has been shown to improve stability of miniprobe and assist in large surface area scanning on other applications necessitates evaluation on PG.
Ultimately, what we need is an integrated pCLE system that allows image acquisition of a wide surface area while preserving the image resolution and consistency of miniprobe deployment so that panoramic evaluation of the surface vasculature could be systematically and objectively evaluated with reference to post-operative PG function.

6.4.7 Conclusion

This study is to our knowledge the first to report on the use of pCLE to obtain high-resolution, real-time in situ images of PG vascular morphology intraoperatively. Characteristic features of vessel wall, erythrocyte flow and variations in flow rate were deducible on post-hoc evaluation. The absence of vessel flow particularly on larger sized vasculature may serve as a 'red flag' warning sign of an impending PG ischemia. Further evaluations to examine its relationship with coexisting adjacent vessel flow over a broader surface area are warranted. To this end, strategic modifications of the current pCLE system is required to provide the optical means to systematically evaluate its full potential as an intraoperative imaging adjunct to facilitate decision-making pertaining to autotransplantation of non-viable PG.
Chapter 7: Conclusions

7.1 Achievements of this thesis and the foundations for future work

Prior to this thesis, the role of pCLE imaging as an intraoperative in situ imaging tool for surgical applications had been sparsely investigated. With the exception of tumor margin assessment in neurosurgery (199, 200), its role in general surgery applications was confined to ‘proof of concept’ studies which demonstrated the feasibility of image acquisition on a variety of tissues and organs with no clear clinical application in mind (97, 98). The overarching aims of this thesis are evaluation of feasibility and practicalities of pCLE image acquisition in pre-defined clinical applications. Chapter 2 began this journey through systematic expellment of the myth that pCLE imaging was confined to epithelial surfaces of intact endoluminal surfaces. This was shown to be not the case as pCLE image acquisition was found to be feasible on soft tissue surfaces albeit in a slightly blood-stained environment. Additionally, the use of a sterile transparent drape to maintain sterility of the intraoperative field did not impede the clarity or consistency of image acquisition of tissues. Instead, it facilitated probe movements by acting as a smooth interface between probe tip and tissue surface, hence avoid the dragging effect that has long deterred meaningful mosaics to be built. These findings laid the foundations on which subsequent Chapters were built upon.

Chapter 3 provided a detailed description of common morphological features seen on breast tissues and subdivided these into neoplastic and non-neoplastic morphology with the help of corresponding histology slides as the gold standard for comparison. While some findings reaffirmed old information such as the role of topical AH as a nuclear-staining agent, we discovered numerous morphological features that held pivotal information that distinguished neoplastic from non-neoplastic breast tissues. The interpretation of pCLE images by clinicians with little or no histopathology background remained a contentious topic and often evoked feelings of anxiety as to who should be assigned to interpret these images. We anticipated these concerns to be relevant in real-time intraoperative breast cavity
wall margin assessments. To this end, we created a pCLE classification to facilitate a systematic approach in pCLE image interpretation and validated this in a structured assessment of 17 surgeons and histopathologists. Consistent with studies from endoluminal applications, we have shown that the pCLE pattern recognition skills were readily learnt and that the interpretation accuracies from both groups were comparable. The key chapter findings here were that neoplastic features were readily distinguishable from those acquired from non-neoplastic tissues and that the relevant pattern recognition skills were readily learnt by surgeons with little or no histopathology background.

Given that intravenous SF is the most commonly used contrast agent in pCLE imaging of the endoluminal tract, Chapter 4 explored whether it had a diagnostic role in the aforementioned breast cavity wall margins assessment. Consistent with previous studies, SF was well tolerated with no adverse events. However, the lack of nuclear visualization rendered assessment of tissue cellularity impossible on intravenously stained ex vivo specimens. Consequently, there was no role for intravenous SF as the sole staining agent for differentiation between neoplastic and non-neoplastic morphology. However, the staining uniformity conferred by intravenously administered SF rendered construction of pCLE image mosaics comparatively easier as evidenced by a significantly higher mean longitudinal length of pCLE image mosaics in tissues stained with SF with or without AF as compared to those with AF alone. In the presence of tissue deformation, these subtle advantages that SF conferred could potentially have a significant impact in facilitating the consistency of pCLE image acquisition. Nevertheless, these warrants intraoperative evaluation and an algorithm have been proposed to include the envisaged timing of SF administration to ascertain its intraoperative role.

The findings from Chapter 3 provided the building blocks for Chapter 5 where evaluation of the role of pCLE in parathyroid surgery was investigated utilizing topical AH as the fluorescent agent. Given the lack of nuclei visualization (shown in Chapter 4), we decided not to investigate the role of intravenous SF in Chapter 5. This chapter provided novel morphological descriptions of PG and adjacent non-PG tissues using histology as the gold standard for comparison. The morphological architecture depicted by PG tissues were markedly distinct from that of non-PG tissues and were
readily distinguished by surgeons with little or no histopathology background following a similar pattern recognition training session used in Chapter 3 but adapted to the relevant tissue entities. In the context of parathyroid surgery for non-localized hyperparathyroidism and re-operative surgery, these findings sparked a new enthusiasm pertaining to its potential role in facilitating tissue differentiation as an alternative to frozen section and IOPTH assay which has its own well-known limitations. The use of pCLE as an extension to the surgical eye during parathyroid surgery warrants intraoperative evaluation and the findings from this Chapter will provide the baseline information required for tissue-specific pattern recognition in the upcoming studies.

Chapter 6 summarized perhaps one of the most intriguing intraoperative pCLE findings discovered to date. In a feasibility study on 20 patients utilizing intravenous FS as the contrast agent, the vasculature of PGs were readily visualized in real-time using a sterile-draped pCLE probe. The findings of this study re-affirmed those reported in Chapter 2 and provided a valuable insight on the structural and dynamic constituents of PG vasculature. Consistent with endoluminal mucosal vascular architecture, vessels of various sizes and shapes including cellular constituents such as red blood cells were distinguishable from non-vascular components. The morphological characteristics of viable and non-viable PGs were investigated in a well-designed study through careful preservation of the vascular pedicle for the former and following PG devascularisation (post-excision) for the latter. The interobserver agreements of viable and non-viable vascular architecture were shown to be substantial amongst six assessors with minimal pCLE image acquisition experience. Equipped with these pattern recognition skills, the next obvious step would require a well-powered intraoperative in situ study that assesses the correlation between these findings with post-operative PG function in the form of post-operative corrected calcium and PTH levels. However, the preliminary findings of well-vascularised PGs with absence of post-operative parathyroid function raised the possibility that the regions imaged might not be truly representative of the overall vascular supply of the PG and thereby conclusions pertaining to PG viability could not be reliably deduced at this stage.
7.2 Bridging the gap between novel discoveries and clinical applications

While the physical properties of pCLE was designed clearly to allow flexibility and accessibility in endoluminal applications, these confer similar advantages in open surgery where image acquisition of tissues could be obtained by direct deployment of probe tip surface based on the surgeon’s judgement without having to engage in the cumbersome manouevres of multiple probe position re-adjustments through a third-party stabilizer or deployer. However, there are consequences as a result of the current state of miniaturisation. The field of view of image frames reduces with decreasing probe diameter, an inevitable optical trade off. Consequently, we rely on mosaicking to create field of views that are representative of the tissues imaged. These warrant deployment of the pCLE probe with precision and accuracy. In the presence of intraoperative time constraint and soft tissue deformation, technological adjuncts to promote stability and precision are warranted. It is inevitable that the transition of pCLE from a ‘research tool’ to ‘clinical device’ require integration with concurrently developed medical engineering tools that are specifically adapted to augment pCLE image acquisition in challenging intraoperative environments. Given the potential pCLE imaging hold as evidenced from the findings of this thesis, our next research priority should involve systematic evaluation of technological adjuncts to augment intraoperative usage of pCLE. These should include systematic assessment of the ideal optical trade-offs for pCLE intraoperative deployment and integration of robotic assisted technologies and pervasive sensing to promote accuracy, precision and reproducibility (Figure 7.1).

The concept of integrating an imaging tool with technological adjuncts is best described in the example of endoscopic ultrasound (EUS) and the adoption of fine needle aspiration (FNA) (201, 202). First described in 1980, EUS was initially an experimental research tool, but over the years, the combination of FNA with EUS has transformed its role to a validated diagnostic tool in a variety of clinical applications such as the diagnosis of indeterminate pancreatic cysts and gastrointestinal stromal tumors (GIST) (202-204). It remains to be seen whether recent advances in surgical robotics and microprecision tools would augment the full potential of pCLE imaging and thereby warrants evaluation.
Figure 7.1 This flow-diagram depicts the three phases of research development of pCLE image towards real-time applications in surgery. This thesis established the foundations whereby promising intraoperative applications were discovered as shown in Phase 1 (highlighted in yellow). Additionally, it laid the building stones for Phase 2 which comprises of systems evaluation using technological adjuncts to augment image acquisition. This constitutes our next research priority prior to embarking on the relevant clinical trials.
7.2.1. Adaptive optical imaging

The ideal trade-off between pCLE probe diameter and field of view warrants careful consideration of the following tissue-specific and morphology-specific factors:

- **Surface area of coverage (tissue-specific factor)**
  
  For applications pertaining to breast cavity wall assessment, the surface area of a quadrant of interest presents a significant challenge to cover within the time-constraints in an operating theatre. A potential solution to this might include initial scanning with larger field of view frames for detection of foci of hypercellularity. The image processing software should offer real-time options of magnifying the foci of concern whilst preserving the resolution required for detailed morphological assessments. This inevitably warrants a large diameter pCLE probe (Figure 7.2).

- **Accessibility and degree of angulation (tissue specific factor)**
  
  The challenges pertaining to accessibility of breast cavity wall assessment are unique in that in situ angulation of the probe tip is required to achieve perpendicular contact against the tissue surface of the lateral cavity walls. The degree of angulation allowed is dependent on the number and physical properties of optical fibres contained within the pCLE probe. A larger diameter confers ability to image a wider field of view. However, that could potentially restrict the degree of angulation. Hence, an assessment of pCLE probe size, degree of angulation and resolution threshold are warranted, perhaps in a simulated breast cavity model in the first instance.

- **Morphological requirements of tissue differentiation (morphology-specific factor)**
  
  The degree of resolution required for detection of neoplastic foci during initial breast cavity wall scanning differ from those required for vascularity assessment of PGs. The latter warrants high resolution images to ascertain the presence of vascular flow albeit a wider field of view simultaneously. An objective assessment with small-scale increments in the field of view and amount of diagnostically important morphological details preserved might provide a solution to establishing an optimal tradeoff.
Figure 7.2 A selection of endomicroscopy probes to be tested for optical tradeoffs in the aforementioned surgical applications. (a) A bare-tipped 10k core, 0.6 mm diameter Sumitomo bundle; (b) A 30k core, 0.5 mm diameter Fujikura bundle with an integrated x2 magnification distal lens assembly (1.4 mm in diameter); (c) an ultra-flexible 17,000 core Schott leached imaging bundle with a diameter of 1.2 mm; (d) a semi-rigid, 100k core Fujikura fibre bundle, 1.7 mm in diameter; and (e) a 50k core Schott rigid image conduit, 3.2 mm in diameter. [Image courtesy of Dr Michael Hughes, Research Associate, The Hamlyn Centre, Imperial College London]

7.2.2. Robotic technologies and pervasive sensing

Efforts have also begun integrating the use of a light-weight 7 degrees-of-freedom compliant robotic manipulator to create simultaneous CLE mosaicing and localisation in a simulated breast cavity (Figure 7.3). The pCLE probe tip position produced by the robot was combined with image registration was capable of generating a functional three-dimensional map of the simulated cavity surface [18]. A hand-held force-adaptive instrument was integrated into the robotic arm to aid maintenance of a constant predetermined contact force against the tissue surface (95, 96). It automatically adjusts the forces exerted based on the regularity of the tissue surface it encounters during cavity scanning thus improving the consistency and quality of CLE images acquired. The
Kinematic redundancy of a light weight robot is a key advantage in that it offers enhanced dexterity and flexibility, allowing maintenance of a consistent perpendicular positioning of the CLE probe tip against the tissue surface. Based on our early experience, amongst the perceived benefits of a robotic manipulator include providing stable positioning and movement precision of the CLE probe while eliminating the aforementioned global registration problem given that the CLE images are registered to the manipulator frame of reference i.e. simultaneous tracking of the probe position.

Figure 7.3 (a) Robotic manipulator system set-up comprising of a light weight KUKA© robot with the force adaptive control instrument mounted at the end-effector of the robotic arm with CLE image mosaic construction obtained from a simulated breast cavity; (b) During CLE image acquisition, the position of robot in 3-dimensional space were recorded and surface reconstruction with mapping of the CLE image mosaic were mapped to its corresponding location in the simulated breast cavity. [Adapted from Simaiaki et al, (95)]

7.3 Alternative technologies for real-time tissue diagnostics

Whilst this thesis embodied numerous novel and exciting pCLE results, albeit preliminary at this stage, it is important to be cognizant of the various emerging real-time tissue diagnostic technologies within which pCLE works. Of note, two competing technologies have recently stimulated significant interest and were developed during the timeline of pCLE expansion.
7.3.1 Intelligent surgical knife

One of the most promising emerging technologies that garnered significant scientific and media attention of late is the “intelligent surgical knife” (iKnife). This technology is derived from the coupling of electrosurgery with rapid evaporative ionization mass spectrometry (REIMS) for real-time tissue diagnostics (205). Electrosurgery is a technology invented in the 1920s that is commonly used in surgery today. Electrosurgical knives use an electrical current to rapidly heat tissue, cutting through it while minimising blood loss. In doing so, they vaporise the tissue, creating aerosol that is normally sucked away by extraction systems. REIMS on the other hand is an emerging technique that allows near–real-time characterization of human tissue in vivo by analysis of the aerosol released during electrosurgery. In a recent study on freshly excised human tissue samples by Balog et al, iKnife was shown to be able to differentiate accurately between distinct histological and histopathological tissue types, with malignant tissues yielding chemical characteristics specific to their histopathological subtypes (205). Tissue identification via intraoperative REIMS matched the postoperative histological diagnosis in 100% (all 81) of the cases studied (205). Whilst these results are promising, it has yet to be shown whether these results are reproducible in in vivo settings and its potential impact on patient oncological outcomes warrants prospective evaluation.

The unique advantage that pCLE possesses is that it obviates the need to engage in cauterisation of tissues to yield diagnostic information. It merely presents valuable information pertaining to morphological architecture in its original state through high powered magnification and contrast agents to augment the appearances of its cellular constituents. In essence, it allows the tissues to retain its cellular and architectural integrity without having to go through the process of tissue destruction. Evidently, pCLE allows prospective evaluation of the specific area of the tissues analysed following the completion of its image acquisition. However, the process of obtaining wide breast cavity surface area coverage might pose significant challenges for pCLE imaging. Given that the breast cavity walls were created by electrosurgery in the first place, it is envisaged that continuous monitoring of aerosol produced during tumour excision might provide valuable adjunct information by iKnife technologies.
It is worth reminding that the envisaged intraoperative applications of pCLE imaging include parathyroid and thyroid surgeries. It does not appear that there is a clear role for iKnife in either of these applications. The diseased PG needed to be localised first (where the problem lies in parathyroid surgery) before electrosurgery is used on them. Evaluations on vascularity of PGs during thyroid surgery warrants meticulous preservation of its blood supply and destruction of tissues from electrosurgery will not generate any relevant information pertaining to the viability of PGs.

### 7.3.2 Optical coherence tomography

Optical coherence tomography (OCT) is an optical imaging technique that provides high resolution, real-time and multi-dimensional microscopic images of subsurface tissue structure. It is the optical analogue to ultrasound imaging but utilises near-infrared light waves instead of sound waves to create images. One of the key advantages of OCT when compared to pCLE is the former’s ability to provide superior penetration depth of up to 1-2 mm. This depth is not possible to achieve with pCLE as increasing overlying sections of tissue constituents will reduce the clarity of images and cause more image artefacts.

The ability of OCT to differentiate various breast tissue entities was described in a landmark study by Nguyen et al which showed that OCT has the potential to generate high quality images similar that of the histopathological controls (206). Whilst these results are promising, the OCT images were generated at a lateral resolution of 35 μm (far lower than that of 1 μm generated from pCLE). The trade-off for this would be its larger field of view on a single frame (1 cm²). These optical characteristics might facilitate wider surface area coverage in the context of breast cavity scanning but the reduction in the magnification and clarity of stromal and glandular characteristics might render occult and inconspicuous tumour foci difficult to be identified. From our pCLE experience, subtle and loosely dispersed neoplastic deposits such as those often found in invasive lobular carcinoma warrants high magnification imaging to ascertain. Similarly, evaluation of luminal cellularity in glandular tissues warrants detailed assessments to differentiate groups of acini from ductal carcinoma in situ. As
highlighted in the earlier chapters, what is needed are systematic evaluations on the ideal optical trade-off pertaining to the demands of specific applications are required for both pCLE and OCT.

Recently a study Conti de Freitas et al in Massachusetts, Boston, showed that OCT images from tissues obtained at thyroid and parathyroid surgeries correlated well with histopathology and participants in their study were capable of recognizing and differentiating neck tissues encountered during thyroid and parathyroid surgeries (207). A more recent study by Sommerey et al showed that OCT was capable of distinguishing between parathyroid, thyroid, and adipose tissue (208). However, an accurate differentiation between parathyroid tissue and lymph nodes was not possible (208). The authors attributed these findings to problems handling the endoscopic probe intraoperatively (208). We envisaged similar challenges with the pCLE probe and we therefore support the need for further refinement of these technologies to augment the surgical ergonomics. It is important to note that the requirements for highly magnified images of parathyroid morphology are not as critical as that of breast tissues because the former (when diseased), often adopt a more uniformly hypercellular architecture which should be deduced easily on low power magnification. For thyroid surgery applications, there has yet to be any data pertaining to the ability of OCT to provide real-time dynamic information pertaining to vascular flow and therefore no conclusions could be drawn at this stage.
7.4 Closing comments

Currently pCLE holds the potential to yield useful information that could guide decision-making during surgery. It is a niche that has yet to be explored in the aforementioned surgical applications. It is evident that its present state is not best optimised for use intraoperatively. Perhaps, co-registration of technological adjuncts could provide the solution to augment its potential and applicability in an increasingly time-constraint operating theatre. A systematic approach to investigate the optimal trade-offs between the optical resolution requirements of tissue morphology visualization and deployability of pCLE probe holds the key to successful clinical translation. It also undoubtly requires an economical, robust and intelligent platform equipped with the flexibility to cater for tissue surface deformation and precision mechanisms that generates accurate spatio-temporal localisation in real-time to aid intraoperative decision making.
References


171


139. 2015 hwmcecp-c-d-e-faotF.


