

# Establishing imaging protocol for evaluation of breast cancer margins using high-speed confocal endomicroscopy and methylene blue

Khushi Vyas, Michael Hughes and Guang-Zhong Yang

The Hamlyn Centre for Robotic Surgery, IGHI, Imperial College London, UK

[k.vyas13@imperial.ac.uk](mailto:k.vyas13@imperial.ac.uk)

## INTRODUCTION

Breast cancer is one of the leading causes of cancer related deaths worldwide, accounting for about 14% of all cancer related female deaths. The first line of treatment is surgical excision of malignant lesions either by removing the whole breast (mastectomy) or by removing the cancerous lump and a margin of healthy tissue around it (lumpectomy or breast conserving surgery, BCS). BCS is often the treatment of choice for patients with early-stage breast cancer, as it allows for complete tumour excision while still maintaining acceptable cosmesis. However 20-30% of BCS patients require one or more re-operative interventions to obtain oncologically clear resection margins [1].

### *Endomicroscopic imaging of Breast cancer margins*

Imaging systems such as fiber-bundle endomicroscopes, which are capable of performing *in vivo* and real-time optical biopsy, could play a critical role in allowing positive margins to be identified intraoperatively. Preliminary studies into the morphological assessment of breast cancer tissues have been carried out using probe-based confocal laser endomicroscopy (pCLE) at a wavelength of 488 nm. This allows imaging of fluorescent dyes such as fluorescein (intravenous), acriflavine (topical) and proflavine (topical) [2]. Acriflavine allows staining of cell nuclei (unlike fluorescein), but a potential risk of mutagenicity has been identified. For successful clinical translation to *in vivo* cellular imaging it is important to identify fluorescent dyes that are safe for in-human use.

In this work, we develop an in-house high-speed line scan confocal endomicroscopy system to operate at 660 nm wavelength. At this wavelength we can obtain fluorescence images of freshly-excised human breast tissue stained with methylene blue, an FDA approved dye which is already used clinically for intra-operative sentinel lymph node mapping in breast cancer surgery.

## MATERIALS AND METHODS

### *Imaging system*

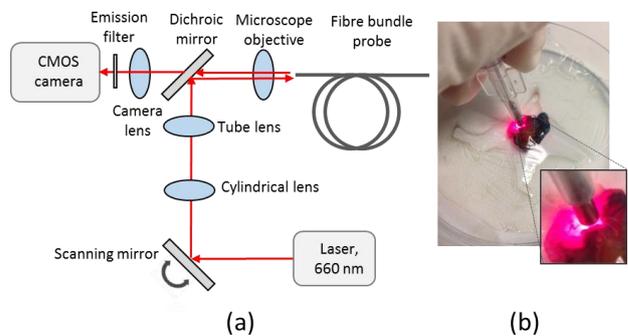
For this study we have converted a high-speed virtual-slit line-scanning confocal endomicroscopy system [3] to operate at 660 nm wavelength (Fig. 1(a)). It uses a cylindrical lens to focus 660 nm light emitted from a laser-diode into a line, which is scanned over the tissue via a flexible fiber-bundle based probe. Returning fluorescence is imaged onto a monochrome rolling shutter CMOS camera (Flea3 FL3-U3-13S2M-CS). The

rolling shutter acts as a virtual detector slit, rejecting most of the out-of-focus fluorescence, leading to optical sectioning at frame rates of up to 120 Hz [3].

### *Patient Data*

The study comprised 8 non-neoplastic human breast tissue specimens freshly excised from 2 patients who underwent breast surgery in the months of March and April 2016. Written informed consent was obtained from the patients using the Imperial College tissue bank ethical protocol (R-12047). For this preliminary study, tissue slices that contained non-neoplastic adipose tissue and stroma (as examined by histology) were selected.

The specimen cut-outs (~10x10mm) were first immersed in methylene blue solution at four different concentrations of 0.1%, 0.2%, 0.5% and 1%. The fluorescent agent was left to stain the tissue for 30 s. Following immersion, the cut-outs were gently rinsed with water to wash off excess fluorescent agent for 1 minute and imaged immediately. A representative tissue specimen is shown in Fig. 1(b).



**Fig 1.** Experimental set-up showing (a) Schematics of the optical system and (b) image of the fiber-bundle probe examining methylene blue stained breast tissue

### *Image acquisition and processing*

The tip of the endomicroscope probe (Cellvizio Coloflex UHD probe, Mauna Kea Technologies), which contains a 30,000-core Fujikura fiber bundle (FIGH-30-650S), was gently pressed onto the tissue surface. Images were obtained in real-time and stored digitally in a prospectively maintained database of images and corresponding histology. At the end of the imaging session, excess fluorescent dye was gently wiped off the surface of the tissue and the tissue was returned to histology for routine analysis. A pre-processing step for removing the pixelation artefacts produced by the fiber cores, using Delaunay triangulation, and a post-

processing step for video mosaicking to create larger field of view images, using a two-way fast normalized cross-correlation (NCC) algorithm, were implemented.

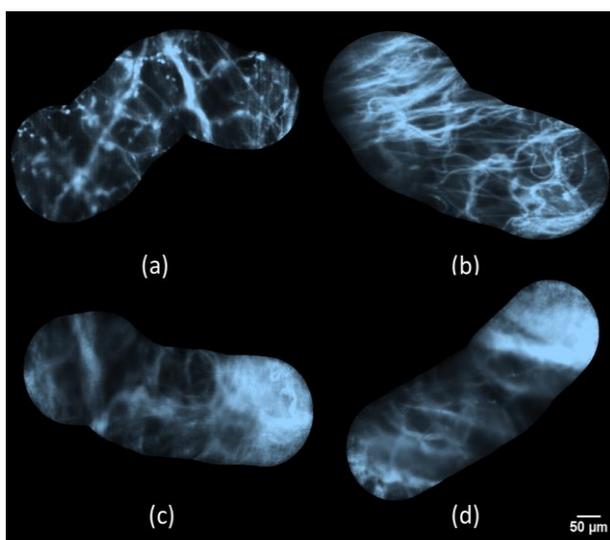
### Image quality assessment

Morphological features were identified following the taxonomy of pathologies for normal and neoplastic breast tissue that was developed in our previous study [2] and the image quality was assessed from the visibility of those features in the reconstructed mosaics. The mosaics were scored '1', '2' or '3' based on the clarity with which the tissue structure was visualized (where '1' - almost invisible structure, '2' - partly visible and '3' - clear classification of tissue morphology). The rationale for this categorization was that, if the tissue is stained non-specifically, different tissue structures such as fat cells, nuclei and connecting fibers would all be stained uniformly, thus making it difficult to identify and distinguish them. This would be reflected by an overall reduction in mean image contrast of that image.

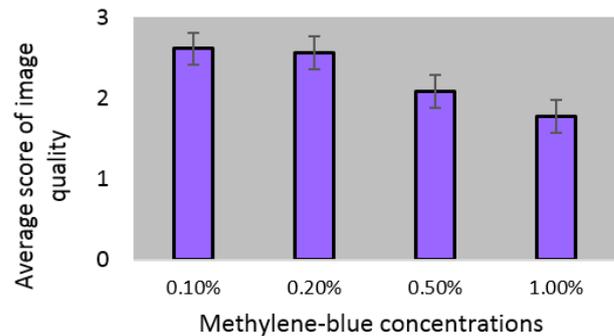
## RESULTS

The main types of normal breast tissue features present in all the 8 specimens analyzed were adipose tissue and collagen fibers in the stroma. All the specimens were histologically confirmed non-neoplastic. On average 3 imaging tests were performed on each sample and a total of 240 image mosaics were generated. Endomicroscopy images of specimens at different staining concentrations are shown in Fig.2.

In fluorescence endomicroscopy images, the adipose tissue appeared as well-defined and uniform black non-fluorescent oval or polygon shapes with hyper-fluorescent borders and sparsely populated nuclei as bright spots along the borders. The fibrous connective tissue appeared as well-defined hyper-fluorescent bundles of elastic wavy fibers with sparse oval bright spots corresponding to fibroblast nuclei in the stroma.



**Fig. 2** Representative endomicroscopy mosaics of breast tissue stained with methylene blue at (a) 0.1%, (b) 0.2%, (c) 0.5% and (d) 1.0% concentration. The elastic fibers and nuclei are clearly visible in (a) and (b).



**Fig. 3** Effect of methylene blue concentration on endomicroscopy image quality

Fig. 3 shows the overall scores from the assessment of tissue structure visibility in endomicroscopy mosaics stained at different methylene-blue concentrations. For 0.1% and 0.2% of methylene blue, tissue structure is clearly visible and nuclei are visible as bright spots. For higher concentrations of 0.5% and 1%, even though adipose tissue was partially visible, the hyper-fluorescent, thread-like connective fibers were not identified and the stoma appeared uniformly bright making it difficult to distinguish nuclei.

## DISCUSSION

This work shows the potential use of high-speed line scan confocal endomicroscopy operating at 660 nm to evaluate radial margins during BCS in real-time. Clinical trials have demonstrated the suitability of methylene blue for endomicroscopy imaging of human airways using point-scanning pCLE, typically having frame rates on the order of 12 fps (Mauna Kea Technologies). With our system, we can achieve frame rates up to 120 fps, an order of magnitude improvement which helps enable large-area imaging by mosaicking while maintaining high resolution.

Existing literature on endomicroscopy imaging of lung nodules suggests a methylene blue concentration of 0.1%. Our preliminary study indicates that concentrations of 0.1% and 0.2% give optimal results for freshly excised normal breast tissue specimens. Work is on-going to examine the tissue morphology of benign and neo-plastic breast tissue using these different methylene blue concentrations, and obtaining objective assessment by trained pathologists. This work will inform potential future studies using 660 nm confocal endomicroscopy directly in the breast cavity.

## REFERENCES

- [1] P. Ananthkrishnan et al., "Optimizing surgical margins in breast conservation. International Journal of Surgical Oncology, 2012, Article ID 585670."
- [2] Chang, Tou Pin, et al. "Imaging breast cancer morphology using probe-based confocal laser endomicroscopy: towards a real-time intraoperative imaging tool for cavity scanning." Breast cancer research and treatment 153.2 (2015): 299-310.
- [3] Hughes, M. et. al. "Line-scanning fiber bundle endomicroscopy with a virtual detector slit." Biomedical Optics Express 7.6 (2016): 2257-68.