Dual mode fibre bundle confocal endomicroscopy: combining reflectance and fluorescence imaging

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ABSTRACT

Commercial endomicroscopes operate in fluorescence mode only and so require the application of contrast agents. As an alternative, we describe a fibre bundle confocal endomicroscope which acquires simultaneous and co-registered fluorescence and reflectance mode images. A combination of polarisation selection and refractive index matching is used to minimise back-reflections from the fibre bundle. We show preliminary results from the system using phantoms and tissue samples.

Keywords: endomicroscopy, confocal, endoscopy, fluorescence, reflectance, dual-mode, optical, biopsy

1. INTRODUCTION

Confocal endomicroscopy[1] offers real-time, cellular-scale visualisation of human tissue. Modern endomicroscopy probes are small enough to pass along the instrument channels of endoscopes, allowing minimally invasive microscopy deep inside the body. There are many promising clinical applications for this 'optical biopsy' technique, and it could, in some cases, reduce the need for conventional biopsy and histology. Nevertheless, endomicroscopy images are inferior to histological micrographs for a number of reasons, and not least because most histology stains cannot be used *in vivo*. Endomicroscopy for *in situ* applications could therefore benefit from any additional image contrast mechanisms.

The two commercially available endomicroscopes (Mauna Kea Cellvizio and Pentax ISC-1000) operate in fluorescence mode and so require the use topical or intravenous fluorescent contrast agents. Reflectance mode endomicroscopy[2,3] - for which no contrast agent is required - suffers from several drawbacks, and has yet to find significant applications. While in principle reflection-mode imaging does not require contrast agents, in practice the contrast-to-noise ratio is low for many tissues. Fibre bundle endomicroscopes are particularly troublesome to operate in reflectance mode due to the problem of back-reflections from both faces of the bundle[2]. Without recourse to the simple wavelength filtering that is possible in fluorescence mode imaging, removing these reflections requires arrangements involving index matching gels[2], polarisation selection[4] or time-of-flight measurements[5]. Both modalities therefore suffer from their own, individual limitations.

The idea of combining fluorescence and reflectance signals to create a 'dual mode' image is well established in bench-top and dermatological microscopy[6], and was recently extended to endomicroscopy by Cha et al.[7]. However, while their system was sufficient for measuring the efficacy of gene transfection *in vitro*, it was unsuitable for use *in vivo*. In particular, it generated only 1 image frame per second, and the fibre bundle was placed in direct contact with the assay without an intermediary lens. While this approach reduced back-reflections from the distal end of the bundle, it also meant that, among other disadvantages, the lateral resolution was limited by the spacing of the fibre cores.

Below we demonstrate a design for an improved dual mode confocal endomicroscopy system with a higher frame rate, an optimised optical train and a microlens-coupled fibre bundle. By providing both fluorescence and reflectance mode images this design seeks to exploit the advantages of both modalities and increase the range of applications for endomicroscopy.

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2. METHODS

The experimental setup is shown in Figure 1. Polarised light from a laser diode (Vortran Stradus, 488 nm, 50 mW) passes through a polarising beamsplitter (Thorlabs PBS051), aligned for maximum transmission, and a dichroic beamsplitter (Semrock RazorEdge 488 nm). The beam is scanned in a 2D raster across the face of a 2 m long fibre bundle (Fujikura FIGH-30-800G) using a scanning system composed of a galvo scanning mirror (Thorlabs GVS011), an 8 kHz resonant scanning mirror (Cambridge Technology CRS), a X10 microscope objective (Edmund Optics #43-903), and a telescope. A glass cover slip and index matching gel (Thorlabs G608N) are placed in front of the bundle, shifting the back-reflection away from the conjugate plane and allowing for its rejection by a confocal pinhole.

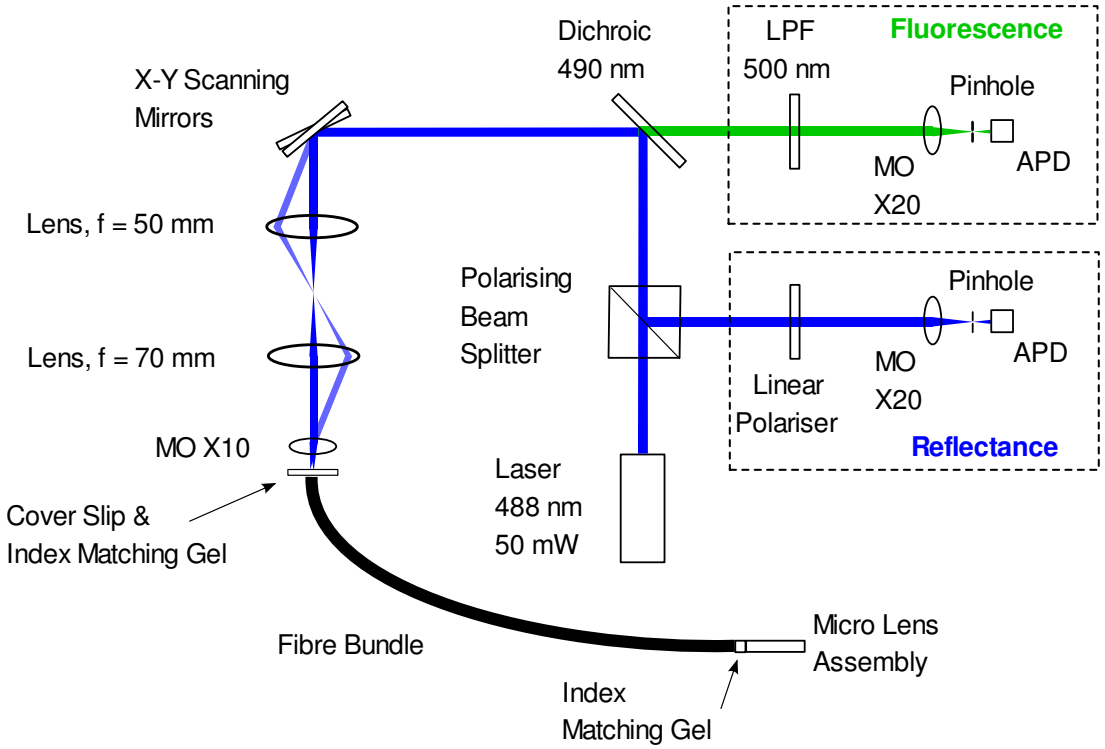

Figure 1. Optical layout for dual mode endomicroscopy system. MO: microscope objective; LPF: long pass emission filter; APD: avalanche photodiode.

At the proximal end of the bundle, index-matching gel couples the light into a GRIN microlens assembly (GRINTech GT-MO-080-0415-488), again minimizing back-reflections. The microlens focuses light onto the tissue with a X1.9 magnification, resulting in a nominal lateral resolution of approximately 2.5 μm and field of view of approximately 400 μm. Reflected light and fluorescence from the tissue is coupled back into the fibre by the microlens and subsequently de-scanned. Fluorescence is transmitted through the dichroic filter into the fluorescence detection arm where it is focused through a 25 μm pinhole (Thorlabs P20S) by a X20 microscope objective, and then passes through a 500 nm long pass emission filter (Thorlabs FEL0500) and onto an avalanche photodiode (Thorlabs APD110A).

Light reflected from the tissue, as well as light back-reflected from the bundle, is reflected by the dichroic to reach the polarising beamsplitter. Light from the tissue will have had its polarisation scrambled, and so approximately 50% will be reflected by the beamsplitter and pass through a linear polariser into the reflectance detection arm. This arm is identical

to the fluorescence arm, minus the emission filter. Light reflected from the proximal face of the bundle will, to a good approximation, have maintained its original polarisation and so not be reflected into the detection arm.

The image acquisition process, which is summarised in Figure 2, is controlled by a software package developed in Labview (NI). The signals from the APDs are digitised and assembled into 500 x 500 pixel image frames by 12 bit analogue to digital converter (ADC) board (NI PCI-5105). The acquisition of a frame is initiated by a software trigger synchronised with a linear voltage ramp applied to the galvo scanner via a DAQ board (NI PCI-6366), and each line is triggered by a pulse from the resonant scanner driver. The total acquisition time for each frame is 63 ms.

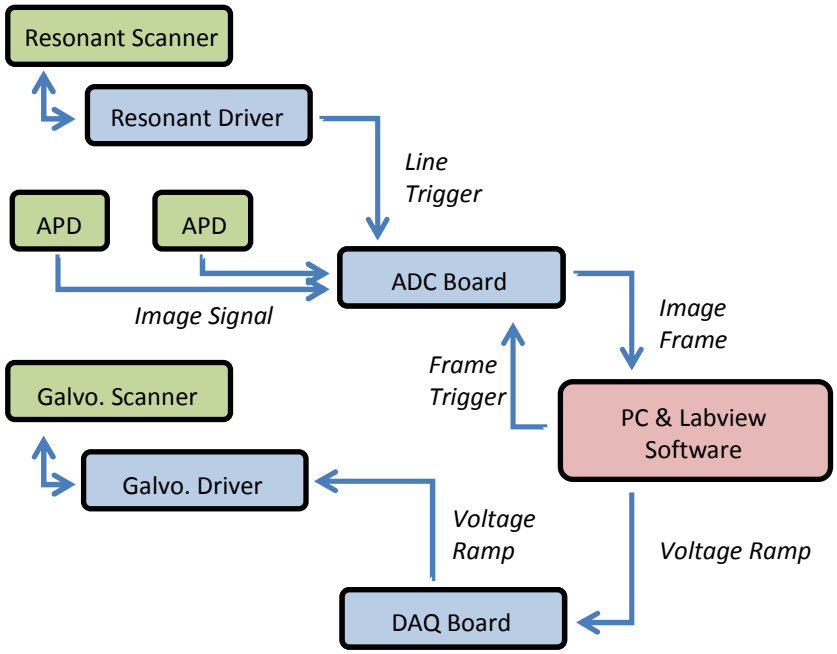

Figure 2. Schematic of galvo control and signal processing scheme. ADC: analogue to digital converter; APD: avalanche photodiode.

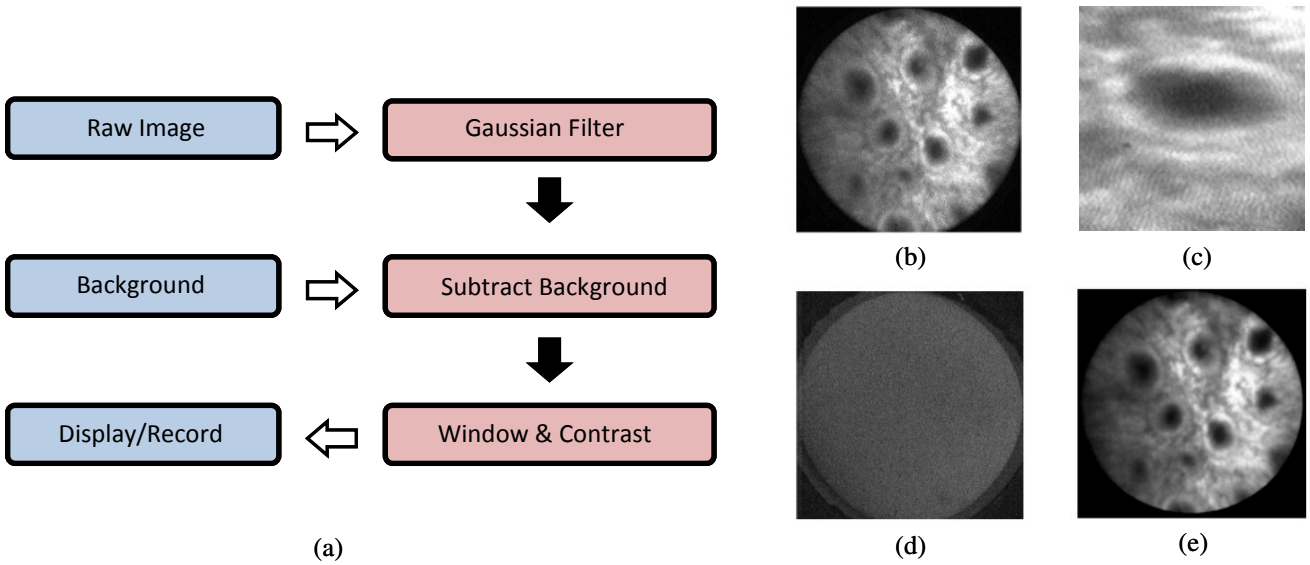

Figure 3. Illustration of real-time image processing scheme. (a) Flow diagram showing processing steps; (b) example raw image (porcine small bowel, fluorescence channel); (c) zoom in on raw image showing honeycomb pattern; (d) background image showing autofluorescence; (e) processed image.

Following acquisition of each image frame, a Gaussian filter (σ = 1.4 pixels) is applied to remove the honeycomb-like structure arising from the discrete fibre bundle cores. More sophisticated methods for removing this pattern have been suggested[8], but in practice we observed only a small difference in image quality. Background corrections for each channel are acquired by averaging 10 frames; these are subtracted from each subsequent image. Finally, a spatial window is applied to remove the edges of the fibre bundle and the image is auto-contrasted for live display to the user at 10 fps, or for recording as an AVI video file. This sequence is illustrated in Figure 3.

3. RESULTS

Figure 4 shows representative imaging results from a phantom and from *in vivo* and *ex vivo* tissue samples. The images were extracted from videos acquired at 10 frames per second. In all three cases the probe was directly handheld and was not deployed through an endoscope. No additional processing was applied to the images except for colour-coding and resizing.

The left column shows lens tissue paper stained with acriflavine 0.05%. Individual tissue strands are clearly visible in both the reflectance (top, blue) and fluorescence (bottom, green) images. Lens tissue is a highly reflective target, and so a high signal to noise ratio (SNR) in the reflectance image was to be expected in this case. Images taken from the surface of a human finger (centre column) also show high SNR in both channels. *Ex vivo* porcine stomach (right column) shows lower contrast in the reflectance channel due to relatively small variations in refractive index across the tissue. In this case the reflectance signal from the sample was comparable in magnitude to the residual back-reflections from the fibre bundle prior to digital background subtraction. Small pieces of debris can also be seen as black spots in this image.

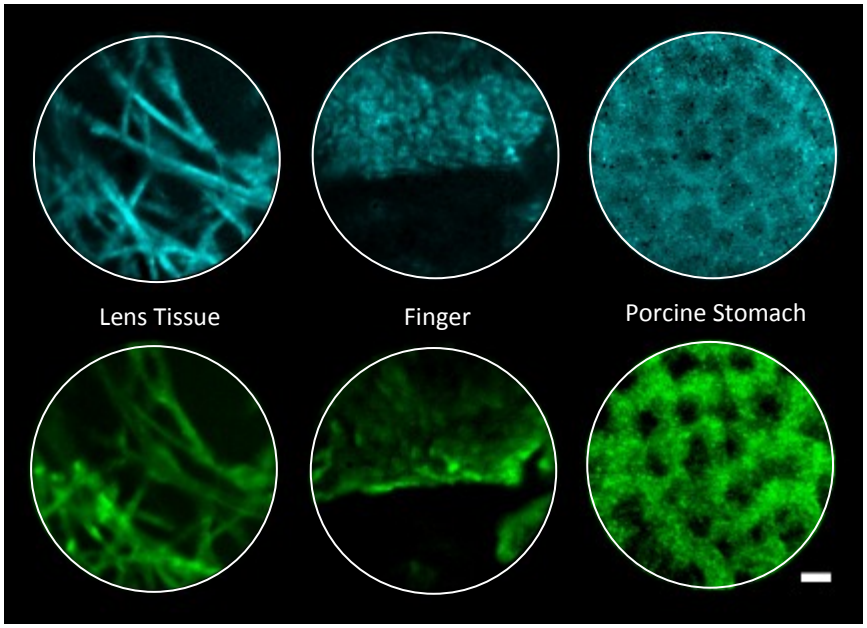

Figure 4. Example dual mode endomicroscopy images. Top row are reflectance, bottom row are fluorescence. Showing (i) lens tissue stained with 0.05% acriflavine, (ii) human finger, (iii) porcine stomach tissue stained with 0.05% acriflavine. Scale bar is 50 μm.

4. DISCUSSION

The addition of a reflection channel to create a dual-mode instrument is a clear improvement over previously reported fluorescence-only systems, and the images above demonstrate that our design is feasible for highly reflecting samples. The optical train is only slightly more complex than that required for a fluorescence-only system, with the main change being the addition of a second detection arm. It would be possible to simplify the optical setup further by using a single spatial filtering system and splitting the two channels just before the APDs, and so the additional costs of such a system are likely to be small. The main engineering challenge lies in ensuring that back-reflections from the fibre bundle are sufficiently attenuated so that the reflectance image is not swamped by noise. Here, we used a combination of three methods: polarisation selection, index-matching and background subtraction, although other solutions may also be feasible.

Despite the combination of approaches, the signal to noise ratio of the reflectance channel remains low, and it is likely that increased rejection of back-reflections will be needed before dual-mode mode endomicroscopy becomes clinically viable. This may be achievable by angle-lapping the fibre bundle ends[2], or by the use of anti-reflection coatings[9]. Some improvement could also be gained by use of contrast agents which alter the refractive index of the tissue. For example, acetic acid has been shown to assist identification of dysplastic tissue using the reflectance channel of a benchtop dual-mode confocal microscope.[10]

Once the back-reflection problem is comprehensively solved, attention can be turned to potential applications. A key advantage of the dual mode over conventional systems lies in its flexibility; it is possible to image stained and unstained tissues within a single procedure, using the same instrument. This would allow a more selective application of topical fluorescent agents to areas of interest. A second advantage arises from use of the reflectance channel for structural and morphological imaging: the fluorescence channel is now made available for functional imaging. This idea has already been exploited somewhat by Cha et al.[7], and there are many other possible applications of fluorescent labeling. Further work involving the study of a wide range of tissues under dual mode endomicroscopy will be required in order to realise this potential.

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