Multiphoton endoscopy

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Motivation

Cancer is the second leading cause of death is the US exceeded only by heart disease. The majority of cancers are of epithelial origin. Early diagnosis of pre-invasive curable epithelial neoplasia can dramatically reduce both the incidence and mortality associated with cancer. Thus, there is a desperate need for highly sensitive and cost-effective screening and diagnostic techniques to identify curable pre-cancerous lesions. Epithelial pre-cancers are characterized by a variety of architectural and morphological features, including increased nuclear size, increased nuclear/cytoplasm ratio, hyperchromasia, and pleomorphism (*1*). In addition, there is increasing evidence of significant changes occurring in the stromal layer at the earliest stages of carcinogenesis. A major limitation of current clinical diagnostics is that morphological and molecular changes associated with carcinogenesis can be assessed only by invasive biopsy. Conventionally, a suspicious lesion is first examined visually and, if warranted, biopsied and examined histologically. Visual examination is based on the subjective experience of the medical practitioner and cannot be considered reliable, whereas, biopsy is not only invasive and expensive, but also time-consuming, which often entails the patient anxiety when waiting for the biopsy results. Moreover, margin determination of the tumour lesion, both lateral and in-depth, is not satisfactory.

The shortfalls of the existing methods call for new non-invasive techniques, capable of *in vivo* cancer diagnostics. Such medical imaging techniques as x-ray computed tomography, single-photon emission tomography, ultrasound, and magnetic resonance imaging have become indispensable tools in clinical environments. They are capable of providing morphological visualisation of tissue lesions on macro (millimetre or sub-millimetre) scale, although not on the cellular level, which is of critical importance for cancer diagnosis. Among functional medical imaging modalities, positron emission tomography is capable of detecting and identifying malignant lesions based on their physiological signatures, but the technique resolution and sensitivity is inadequate to detect cancer at early stages. In addition, positron emission tomography is extremely expensive costing 20-30 million dollars, which makes in unaffordable by average clinics.

Optical medical imaging

Optical imaging applied to early cancer detection represents an attractive alternative to the existing medical imaging due to its non-invasiveness, low-cost, high sensitivity, and micron-scale resolution that allows imaging tissue on cellular and sub-cellular levels. A very successful optical imaging modality, termed endomicroscopy, has been most recently demonstrated by OptiScan, an Australia-based company (presently, in partnership with Pentax), and is believed to hold a great promise for early *in vivo* diagnosis of epithelial neoplasia in hollow organs, such as the gastrointestinal tract and colon. Endomicroscopy is a form of laser-scanning fluorescence confocal microscopy (FCM) that efficiently excites and detects fluorescence in a tiny focal volume (micron-scale liner dimension), but nowhere else. By rastering a focal spot across the tissue and detecting fluorescence signal originated at the focal spot point-by-point through a matching pinhole in front of the detector, only a micron-thin tissue layer is imaged. This imaging property of discriminating only a thin layer from within the rest of the sample is called “optical sectioning”, and is truly invaluable for biomedical imaging applications. Optical

sectioning allows unprecedentedly clear imaging of tissue subsurface structures, which are not obscured by neighbouring tissue layers.

Even though the concept of confocal microscopy was introduced as early as in 1950-s, it took several decades to implement it, first, in bulk optics in form of FCM, and later in 1980-s, in fibre optics. Nowadays, FCM is a universal and powerful imaging technique, which has been instrumental in a number of discoveries in Life Sciences. Fibre-optic implementation of the confocal microscopy concept has paved a way to endoscopic imaging applications, although the progress has been slow. Among several technological challenges, flexible delivery of laser excitation light to tissue, and; miniaturisation of the focussed beam scanner (raster) to fit it to the existing optical endoscopes, and; high numerical aperture (NA) lens at the fibre distal end for efficient collection of tissue fluorescent response and for attainment of resolution at the cellular level. Flexible delivery of laser light has been realised by employing a single-mode optical fibre (SMF). SMF is terminated by a custom-designed, sophisticated, high-NA objective lens in optical contact with tissue, so that laser light, coupled into the proximal fibre end, is tightly focussed in tissue. The excited tissue fluorescence is collected by the same excitation module lens/fibre, where the SMF fibre distal end serves as a confocal pinhole to reject out-of-focus light, i.e. optical sectioning is again realised. The beam scanner is realised by mounting SMF on two transversally oriented tuning forks.

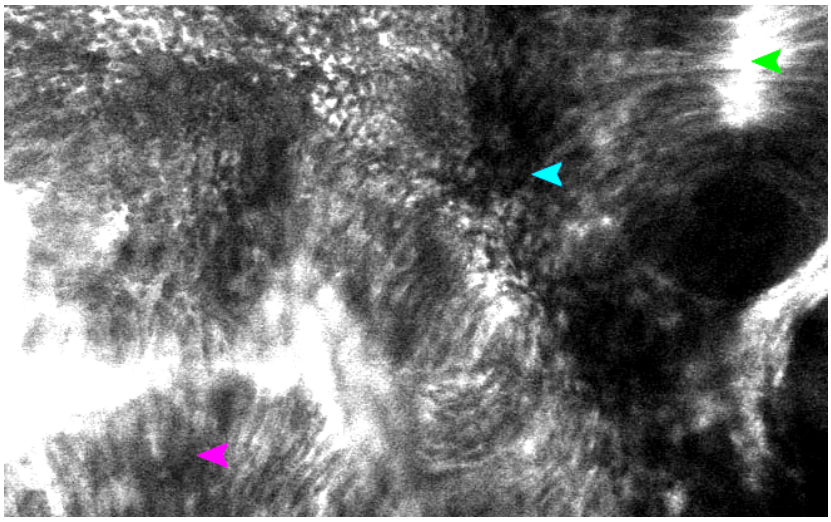

Figure 1. An image of the suspicious polyp acquired by the endomicroscopy system. Gross tissue architecture and cells with dark nuclei, are clearly observable. Note malignant infiltration of cells (top left) in *lamina propria*, that are characterised by different shapes and sizes. (http://www.endomicroscopy.org/casestudies.htm, and explanations therein)

Presently, the endomicroscopy system is at a clinical trial stage showing remarkable promise. The system comprises fibre-based FCM built into the biopsy channel of a conventional endoscope that allows preliminary visual examination of suspicious lesions. Microscopic examination of the lesion is carried out by the fibre-based FCM system following an intravenous injection of fluorescence contrast agent, commonly, fluorescein. A typical *en face* image of the subsurface layer of the polyp, which was diagnosed as malignant, is shown in Figure 1. Clear morphology of the tissue and, especially, cells has been found very helpful for rapid *in vivo* diagnosis with very high (to the best of my knowledge, greater than 90%) sensitivity and specificity. Also, the system capability allows removal of this lesion subject to its subsequent histological examination for confirmation of diagnosis. However, the patient's anxiety is no longer an issue, since the suspicious polyp has been removed.

The endomicroscopy has potential to revolutionise the medical practice of diagnosis and treatment of epithelial cancer of hollow organs. At the same time, this technique is not ideal. First of all, intravenous injection of fluorescence contrast agent is undesirable due to associated patient's discomfort and health risks. Fluorescence-based contrast rendered to tissue and cells is

not easily interpretable, as it varies from tissue to tissue. In some cases, surgery planning and prognosis requires imaging at depths >100 μm that is not accessible by the instrument. Also, FCM, being just an anatomical (not functional) imaging modality, is unable to provide physiological information about a lesion, e.g. its metabolic rate, a discriminating factor of paramount importance.

Multiphoton microscopy

An emerging optical microscopy, termed multiphoton microscopy (MPM), has tremendous potential to overcome the limitations of the existing fibre-based FCM, while retaining its merits (*2*). Since its first demonstration a decade ago (*3*), MPM has been applied to a variety of imaging tasks and has now become a technique of choice for fluorescence microscopy in thick tissue and live animals. MPM offers a key advantage for imaging within strongly scattering biological media: optical sectioning without use of a confocal pinhole for excluding out-of-focus fluorescence. Sectioning arises because the nonlinear, e.g. two-photon, absorption rate peaks sharply at the focus, and tails off outwards. The required high peak intensity values are realised by tight spatial focussing and very short temporal pulses of laser light. In a typical MPM imaging scenario, laser light of average power of 10 milliwatts, focussed to a femto-litre volume, pulsed with a 80-MegaHertz repetition rate with a pulsewidth of 150 femtoseconds, suffices to excite a detectable fluorescence signal. Since fluorescence originates from a focus-limited excitation volume, it is not wastefully discarded by a pinhole – in fact, a pinhole is not used in MPM, leading to a greatly increased collection efficiency, and, hence, greater imaging depth (>200 μm). Other advantages over conventional confocal fluorescence imaging include reduced out-of-focus photobleaching and associated phototoxicity. Also, longer-wavelength excitation laser light (800 nm) penetrates deeper in tissue.

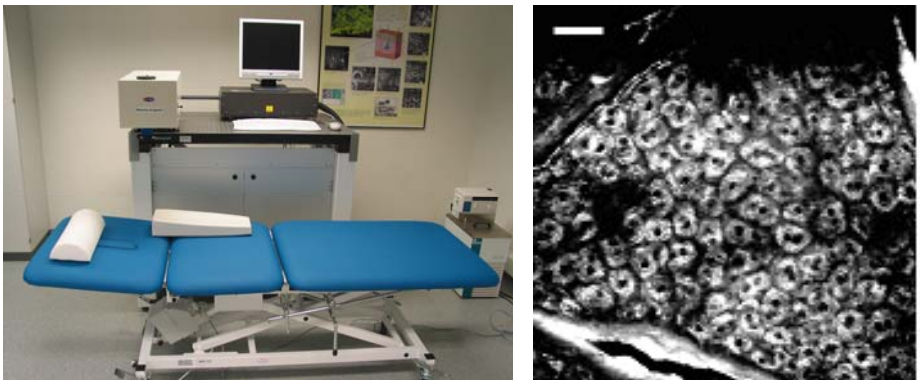

Figure 2. Left panel: "DermaInspect" system (box on the left of the optical table) with a pulsed femtosecond laser (box on the right of the optical table), and with a bedside. Right panel: MPM imaging of the epidermis layer of live skin. Note clearly resolved cells and cell nuclei. Scale bar, 20 μm.
(Courtesy of Prof König, JenLab, Inc.)

Importantly, a spectrum of the multiphoton excitation is, generally, very broad. In context of biomedical imaging, it implies that the excitation light can simultaneously excite a variety of tissue fluorophores, including intrinsic (endogenous) fluorophores, such as keratins, NAD(P)H, melanins, lipofuscin, and porphyrins. In addition, another nonlinear optical process, second-harmonic generation (SHG), accounts for detectability of collagen, which is abundant in skin dermis and internal tissue stroma. Therefore, two-photon excitation and SHG account for clear cellular morphological layout generated by MPM, as shown in Figure 2.

Moreover, accurate analysis of fluorescence lifetime has become possible due to ultrashort pulsewidths generated by a femtosecond laser, and is conventionally realised by the use of a single-photon time correlator in the electronic signal processing circuit (*4*). Therefore, a fluorophore of interest can be accurately identified in the MPM image by analysing its unique spectroscopic and fluorescence lifetime signatures. It is interesting to note that some

physiological conditions of tissue can be assessed employing MPM imaging. For example, NADH, detectable by MPM, is involved in cell energy supply; absence of the NADH signal can be interpreted as the patient's tiredness. Also, skin aging (and skin regeneration, as a result of pharmaceutical treatment) has been found to be quantitatively assessed by detecting a SHG signal due to dermal collagen excited by MPM (*5*). Multiphoton microscopy is, therefore, capable of not only morphological imaging of cells and tissue, but to some degree, functional imaging, such as tissue metabolic activity (based on NADH signal), as well.

Maturity of the femtosecond laser technology has triggered research related to multiphoton microscopy imaging, which has been experiencing explosive growth lately, with a number of relevant publications doubling every year. Multiphoton imaging instruments are now produced by major players in the optical microscopy industry, including Carl Zeiss. At The University of Queensland, we have recently purchased a full-scale multiphoton imaging system, DermaInspect, JenaLab, Germany, certified for *in vivo* imaging of human skin.

Multiphoton endoscopy

It is clear now that implementation of multiphoton microscopy concept in fibre optic for endoscopy applications will provide a new impetus to the field of optical medical imaging. At the same time, development of MPM endoscopy has been slow due to several technological challenges, where the key challenge is flexible delivery of pulsed laser light to imaging point with minimal distortion of the spectral and temporal properties of femtosecond laser pulses. The use of a single-mode optical fibre, as short as 1–10 centimetres, for femtosecond pulses delivery causes severe degradation of their temporal properties, as a result of the combined effects of group-velocity dispersion and self-phase modulation. Group-velocity dispersion in SMF can be precompensated for by use of gratings to provide negative chirping, albeit at power loss, but self-phase modulation is a nonlinear optical effect that causes negatively chirped pulses to undergo spectral narrowing. Thus, prechirped 100-femotsecond pulses of 0.1–1 nanoJoules broaden to 0.2–1 picoseconds after 1 centimetres of travel in SMF (*6*).

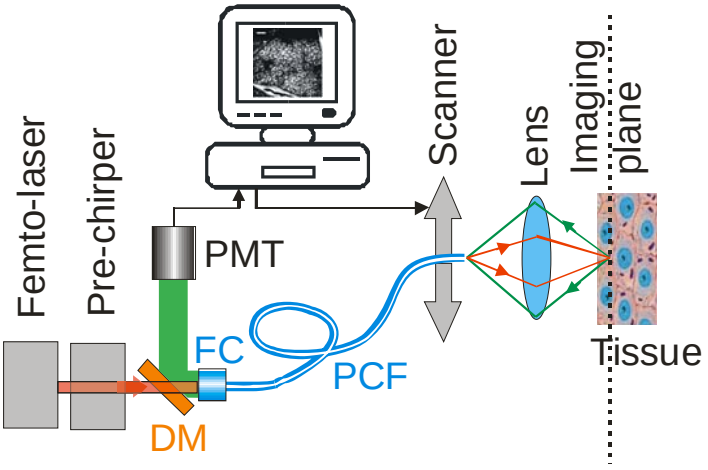

Figure 3. Schematic diagram of the fibre-based multiphoton microscopy system suitable for endoscopic imaging applications. PCF, photonic crystal fibre; FC, fibre coupler; DM, dichroic mirror; PMT, photomultiplier tube.

Among several solutions to mitigate this problem of pulse broadening, Gu *et al*. have proposed the use of a double-clad photonic crystal fibre (PCF) as a means of flexible delivery of the femtosecond pulses to the fibre distal end (*7*). A periodic array of holes in the fibre core and inner cladding of 20- and 165-μm in diameter, respectively, was designed with dual purpose: The femtosecond laser light of a wavelength centred at 800 nm was guided in the fibre core at nearly zero-dispersion condition. Secondly, the fluorescence/SHG signal from the tissue was collected by a high-NA lens, coupled to the inner cladding, and guided to the proximal end of

the fibre. The large diameter of the inner cladding was essential to attain cellular resolution of 1 μm, which has been successfully demonstrated (*7*).

Approach

A schematic diagram of one possible realisation of the MPM system is presented in Figure 3. Light from a femtosecond pulsed laser (Femto-laser) is coupled to a double-clad photonic crystal fibre using a fibre coupler (FC), after passing a pre-chirper. The pre-chirper is employed to impart negative dispersion for compensation of material (positive) dispersion due to optics and tissue. PCF is described in the previous paragraph and in more details in Ref. (*7*). PCF is inserted into one of the biopsy channel of the conventional endoscope. Light delivered to the PCF distal end is coupled out of the fibre and focussed to tissue at a controllable depth by a high-NA objective lens (Lens). The focussed spot is rastered across the tissue by employing a two-dimensional scanning device (Scanner), which can be realised with two microelectromechanical mirrors (MEMS), piezoelectric scanner, or tuning forks. The fluorescence/SHG signal from the tissue is collected by the lens, coupled to the inner cladding of PCF and losslessly guided to the proximal fibre end, where it is collimated by FC, reflected off by the dichroic mirror (DM) towards a photomultiplier tube (PMT). The detected signal is pre-processed by electronics, digitised and fed into a PC for further processing, displaying and archiving. The PC also controls synchronisation of the Femto-laser, Scanner, and PMT. Acquisition of time-gated signals synchronised with the Femto-laser using single-photon time correlator for lifetime imaging is also envisaged.

We identify several critical sub-systems in this project:

1. The most important sub-system is a femtosecond laser characterised by 100-femtosecond laser pulsewidths at a 40-80-MegaHertz repetition rate of average power of several tens of milliwatts. In order to excite major intrinsic fluorophores in tissue, a titanium-sapphire femtosecond laser tunable in the wavelength range of 680 – 1000 nm represents the best choice, since many endogenous fluorophores emit fluorescence in visible, e.g. 470 nm, as NADH. However, high cost of titanium-sapphire systems may restrict wide-spread use of the MPM systems in clinics. A femtosecond fibre laser represents an attractive alternative due to its low cost and good compatibility with the fibre-optic delivery means for endoscopy applications. In particular, the fibre-optic femtosecond laser system Model: EFOA-SH, Avesta, 780 nm seems to be a suitable choice.

2. Design and fabrication of a two-dimensional scanner represents the second most important problem in the realisation of a multiphoton endoscope. Firstly, it needs to be miniature, sized less than 3 mm, preferably, < 1 mm, in order to fit into the conventional endoscope. Secondly, the scanner has to support a scanning rate of 1 frame per second. Considering image size of 1000×1000 pixels, it implies that the faster scanner has to operate at least at a 1-kHz scan rate. Based on the intrinsic optical resolution of 1 μm or better, and allowing adequate sampling, the imaging system field of view should be roughly 200×200 μm^2. Two possible scanner designs are envisaged: two-dimensional angular beam scanning, which results in the focal point lateral scanning in the focal plane of the objective lens; lateral scanning of an entire module “fibre-focussing optics” (based on a gradient-index lens, GRIN).

3. Development and optimisation of fibre-optic delivery of the femtosecond laser light. PCF is available commercially via e.g. Crystal Fiber. However, optimisation of fibre coupling, laser power, and amount of pre-chirping is required in order to achieve the least distorted laser pulses of sufficiently high power at the PCF fibre output.

4. Development of image acquisition software adapted to specific tasks of the endoscopic medical imaging.

At the initial stages of the project, it is reasonable to design and built a MPM delivery and scanning system in bulk optics to characterise separately its key sub-systems. For example, it is the easiest to carry out a feasibility study of the fibre-optic femtosecond laser suitability for imaging of a broad range of tissue endogenous fluorophores using an open-architecture imaging system. Also, optical detection and single-photon time correlation modules will be designed, built, and characterised with their subsequent use in such modular form in the later-stage fibre-based versions of the endoscopy system.

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