# Dual-mode reflectance and fluorescence near-video-rate confocal microscope for architectural, morphological and molecular imaging of tissue

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### Summary

We have developed a near-video-rate dual-mode reflectance and fluorescence confocal microscope for the purpose of imaging *ex vivo* human specimens and *in vivo* animal models. The dual-mode confocal microscope (DCM) has light sources at 488, 664 and 784 nm, a frame rate of 15 frames per second, a maximum field of view of  $300 \times 250 \mu$ m and a resolution limit of 0.31  $\mu$ m laterally and 1.37  $\mu$ m axially. The DCM can image tissue architecture and cellular morphology, as well as molecular properties of tissue, using reflective and fluorescent molecular-specific optical contrast agents.

Images acquired with the DCM demonstrate that the system has the sub-cellular resolution needed to visualize the morphological and molecular changes associated with cancer progression and has the capability to image animal models of disease *in vivo*. In the hamster cheek pouch model of oral carcinogenesis, the DCM was used to image the epithelium and stroma of the cheek pouch; blood flow was visible and areas of dysplasia could be distinguished from normal epithelium using 6% acetic acid contrast. In human oral cavity tissue slices, DCM reflectance images showed an increase in the nuclear-to-cytoplasmic ratio and density of nuclei in neoplastic tissues as compared to normal tissue. After labelling tissue slices with fluorescent contrast agents targeting the epidermal growth factor receptor, an increase in epidermal growth factor receptor expression was detected in cancerous

Correspondence to: Rebecca R. Richards-Kortum. Tel: 713-348-3823; fax: 713-348-5877: e-mail: rkortum@rice.edu tissue as compared to normal tissue. The combination of reflectance and fluorescence imaging in a single system allowed imaging of two different parameters involved in neoplastic progression, providing information about both the morphological and molecular expression changes that occur with cancer progression.

The dual-mode imaging capabilities of the DCM allow investigation of both morphological changes as well as molecular changes that occur in disease processes. Analyzing both factors simultaneously may be advantageous when trying to detect and diagnose disease. The DCM's high resolution and near-video-rate image acquisition and the growing inventory of molecular-specific contrast agents and disease-specific molecular markers holds significant promise for *in vivo* studies of disease processes such as carcinogenesis.

## Introduction

Confocal microscopy is an established optical imaging technique used for high-resolution imaging of cells and tissues. This technique uses a pinhole to reject out-offocus light to provide higher axial resolution and contrast than widefield microscopy and can be performed in either reflectance or fluorescence mode. The diagnostic capabilities of confocal microscopy have been investigated for a number of disease processes in various organ sites, but most researchers have focused on either reflectance or fluorescence confocal imaging separately. However, both methods provide valuable and complementary information about disease processes, suggesting that the combination of techniques may yield improved diagnostic performance. The combination of these two imaging modalities into one system would increase the versatility of the confocal microscope. This would allow images of exogenous contrast agents to be combined with reflectance images of cellular morphology and tissue architecture, to define the location of the contrast agent within the morphology and architecture of the tissue. We have developed a confocal microscope with both reflectance and fluorescence imaging capabilities to image living biological tissues at near video rate to investigate this hypothesis. Although this system can be used to study a variety of disease processes, the applications demonstrated in this paper focus on the detection and monitoring of neoplastic progression.

Diagnosis of neoplastic tissue is typically based on morphological and architectural features that can be assessed with reflectance confocal microscopy. Reflectance confocal microscopy has been used to study the three-dimensional structure and morphology of the human cervix (Collier et al., 2000; Drezek et al., 2000; Collier et al., 2002), skin (Corcuff et al., 1996; Rajadhyaksha, 1999; Rajadhyaksha et al., 1999a, b; Busam et al., 2001; Langley et al., 2001; González & Tannous, 2002; Nori et al., 2004; Gerger et al., 2005) and oral cavity (Watson et al., 1992; Rajadhyaksha et al., 1999a; White et al., 1999; Clark et al., 2003). Several researchers have shown that the morphological features apparent in reflectance confocal images, such as the nuclear density and nuclear-tocytoplasmic ratio, can be used to discriminate precancerous lesions from normal tissues of the cervix and skin (González et al., 1999; Rajadhvaksha, 1999; Drezek et al., 2000; Collier et al., 2002). Fluorescence confocal microscopy has also been used to study the morphology as well as the biochemical composition of human tissues of the colon (Fiarman et al., 1995; Wang et al., 1999; Kiesslich et al., 2004), cervix (Pavlova et al., 2003), oral cavity (Hsu et al., 2006) and skin (Swindle et al., 2003). Researchers have shown that the morphological patterns imaged with fluorescence confocal microscopy after applying fluorescein sodium to the skin and colon corresponded well to the patterns seen in histology (Swindle *et al.*, 2003; Kiesslich *et al.*, 2004).

An exciting new application of confocal microscopy is that of imaging molecular changes within tissues. Many molecular markers are over-expressed in neoplastic tissue compared to normal tissue. These changes can be assessed with molecular-specific optical contrast agents, including metal nanoparticles, nanoshells, organic dyes and quantum dots. Gold nanoparticles and nanoshells, reflective contrast agents, have been used to target specific molecules in cell lines (Sokolov *et al.*, 2003; Loo *et al.*, 2004) and *ex vivo* tissues (Sokolov *et al.*, 2003). Using reflectance confocal microscopy, Sokolov *et al.* have shown that gold nanoparticle–based contrast agents targeting the epidermal growth factor receptor (EGFR) show promise in discriminating between normal and neoplastic cervical tissues (Sokolov *et al.*, 2003). Similarly, organic dyes and quantum dots, fluorescent contrast agents, have been shown to specifically and effectively label molecular targets in cell lines (Jaiswal *et al.*, 2003; Wu *et al.*, 2003; Gao *et al.*, 2004; Lidke *et al.*, 2004), *ex vivo* tissues (Wu *et al.*, 2003; Hsu *et al.*, 2006) and *in vivo* mouse models of disease (Akerman *et al.*, 2002; Gao *et al.*, 2004). Hsu *et al.* used fluorescence confocal microscopy in conjunction with target-specific contrast agents to study the change in EGFR expression in freshly excised neoplastic tissues compared to normal tissues of the oral cavity (Hsu *et al.*, 2006).

Imaging the molecular changes, using molecular-specific contrast agents, as well as the morphological changes may increase our ability to detect precancerous lesions at earlier stages of neoplastic progression. To do this, a system that can image both the contrast agents and the native cellular morphology is needed to place the contrast agent signal in a histologically meaningful context. This type of multimodal imaging can be accomplished by combining reflectance and fluorescence together in a single confocal system. For example, Li et al. recently added a fluorescence channel to a commercially available reflectance confocal microscope to image green fluorescent protein labelled melanoma cells injected intradermally into non-transgenic mouse skin. The fluorescently labelled cells were seen within the context of the host tissue structure that was provided by reflectance images (Li et al., 2005). This type of multi-modal imaging may be very useful in the study of neoplastic progression and the detection of precancerous lesions, as well as other disease processes, where both morphological and molecular information could be assessed together, rather than only looking at morphological information, which is typically the focus for detection and diagnosis.

This paper describes the design of a dual-mode reflectance and fluorescence confocal microscope (DCM) and its application in the real-time, sub-cellular resolution imaging of molecular-specific contrast agents and native tissue architecture and cellular morphology. The DCM was developed to investigate both reflectance and fluorescence contrast in tissue specimens and in vivo animal models with nearvideo-rate image acquisition. Three illumination wavelengths were included in the system for fluorescence imaging of common dyes and quantum dots, reflectance imaging of gold nanoparticles and reflectance imaging of the native tissue backscattering. To study the morphology and molecular markers of interest, the system was designed to achieve a lateral resolution better than 1 µm and an axial resolution better than 2 µm at each of the three illumination wavelengths employed in the system. It was designed with near-videorate image acquisition, a large field of view (FOV) and image tiling capabilities to produce mosaic images of large samples. In addition to the design and characterization of the DCM, this paper demonstrates the system's imaging capabilities in a number of biological samples ranging from cell lines to in vivo hamster oral mucosa, including imaging of ex vivo human oral mucosa tissue.



Fig. 1. Schematic of the optical layout of the dual-mode reflectance and fluorescence confocal microscope.

## Materials and methods

#### Instrumentation

Figure 1 shows a schematic of the DCM design. Figure 2 a shows the middle shelf of the cart that houses the DCM. The middle shelf contains the lasers, shutters that block the laser beams when not in use and laser controls. Figure 2b shows the top shelf of the cart that contains the main optical system.

The DCM has three continuous-wave light sources: an aircooled argon ion laser at 488 nm with a maximum output of 30 mW (National Laser Company, Salt Lake City, UT), a laser diode at 664 nm with a maximum output of 40 mW (Micro Laser Systems, Inc., Garden Grove, CA) and a laser diode at 784 nm with a maximum output of 95 mW (Micro Laser Systems, Inc.). Single-mode optical fibres (Thorlabs, Inc., Newton, NJ) transfer light from the second shelf of the cart

to the top shelf, into the main optical system. The output ends of the fibres are mounted on a computer-controlled translation stage (ILS100PP, Newport Corporation, Irvine, CA) that controls which single laser wavelength enters the optical system. Light exiting the chosen fibre is collimated by lens L1. The collimated beam passes through a variable reflective neutral density filter that is used to control the power incident on the sample. A beam splitter or dichroic mirror directs light towards the sample arm of the system. Two beam splitters and two dichroic mirrors are currently available for use in the DCM. Both beam splitters are 50/50 beam splitters; one for visible wavelengths and one for near-infrared (NIR) wavelengths (Chroma Technology Corporation, Rockingham, VT). Also available are dichroic mirrors for fluorescence imaging at 488- and 664-nm excitations (z488rdc and z658rdc, Chroma Technology Corporation). Each beam splitter/dichroic mirror is mounted on a locking base plate (Newport Corporation),



Fig. 2. Constructed dual-mode reflectance and fluorescence confocal microscope. (a) Middle shelf of the cart, containing the lasers, shutters that block the laser beams when not in use and laser controls. (b) Top shelf of the cart, containing the main optical system.

so that they can easily be interchanged in the system with reproducible positioning.

Within the sample arm, the beam is scanned in two dimensions by an integrated resonant scanning mirror and galvanometer mirror (Electro-Optical Products Corporation, Glendale, NY). The resonant mirror, which produces the line scan, oscillates at 7.9 kHz and the galvanometer mirror rotates at 15 Hz to produce a frame rate of 15 frames per second (fps). Lens pairs L2–L3 and L4–L5 form Keplerian telescopes. Lens pair L4–L5 also expands the beam incident on the back aperture of the objective. The lenses are all achromat doublets with anti-reflection surface coatings. The tube lens (L5) collimates light entering the infinity-corrected,  $40 \times$  magnification, 0.80 numerical aperture (NA), water-immersion microscope objective (Nikon Instruments, Inc., Melville, NY) and the objective focuses the light onto the sample.

Light from the sample is descanned and recollimated by the scanning system and is transmitted through the beam splitter/dichroic mirror into the detection arm. In the detection arm, light is focused by the pinhole lens (L6) through a pinhole and is detected by a photomultiplier tube (PMT) detector. A wheel containing five pinholes ranging in diameter from 20 to 200  $\mu$ m is available in the system so that the pinhole diameter can be adjusted as needed while imaging. The detector is an Electron Tubes analog PMT with a maximum output of 5.4  $\times$  10<sup>10</sup> V/W at 400 nm and 4.5  $\times$  10<sup>9</sup> V/W at 800 nm (Electron Tubes, Rockaway, NJ).

Data from the PMT detector are time-varying voltages, which correspond to the intensity of light from the sample. A MuTech frame grabber card (MV-1000, MuTech, Billerica, MA) was used to digitize the analog signal and convert it to a digital format. Image formation was accomplished by synchronizing the operations of the frame grabber and the scanning mirrors. The scanning mirrors' controller provided two outputs that correspond to the positions of the resonant and galvanometer mirrors; the resonant mirror yielded a sine function output and the galvanometer mirror yielded a sawtooth wave output. A Schmitt trigger circuit was used to detect the zero crossings of these waveforms while ignoring most noise present on the signal. These zero crossings were used to generate the line and frame synchronization pulses for the frame grabber, and images were displayed at a rate of 15 fps on the computer monitor.

The non-linear scanning pattern of the resonant mirror caused the images to be stretched at the edges in the horizontal direction. The distortion in the images was corrected through pixel re-sampling, post-image acquisition, using Matlab (The MathWorks, Inc., Natick, MA). The number of pixels in each line was reduced at the left and right edges of the original image, reducing each line from 760 to 519 pixels.

The DCM stage and controls were designed so that image mosaics could be taken to cover a region of interest (ROI) much larger than the FOV of one frame. The sample stage of the DCM was controlled in three dimensions via the computer. A program was written in LabView (National Instruments, Austin, TX) to control the movement of the sample stage and the capture of images. A calibration utility to determine the image step-size and number of images to acquire for creating an image mosaic covering a user-defined ROI was included in the program. Beginning at one corner of the ROI, the computer controls captured an image, saved the image and moved one step-size to the next imaging position. Images were captured in this method, until the entire ROI had been imaged. As images were recorded, the location of the image within the larger userdefined region (x, y and z location) was appended to the file name automatically. A macro was written in Image J (National Institutes of Health, Bethesda, MD) to create one large image from the individually saved, single-FOV images and to save it with a similar filename. The user first used the Matlab code to resample the images to correct for the line scan distortion, and then ran the Image J macro to build the mosaic from the resampled images.

#### System performance measurement

The lateral resolution of the DCM for reflectance imaging was determined from the distance between the 10 and 90% intensity across the edge of a 1000 line-pair-per-inch Ronchi reflective grating. The lateral resolution of the system was also tested by imaging sub-resolution fluorescent microspheres. Dragon green fluorescent polystyrene spheres, 0.19 µm in diameter (Bangs Laboratories, Fishers, IN), were imaged with 488 nm excitation and 500–550 nm bandpass emission. The optical sectioning thickness was measured by moving a planar mirror through the focus of the system at 0.5  $\mu$ m steps. The optical sectioning thickness was defined as the fullwidth at half maximum of the plot of the average intensity versus axial position. The maximum FOV of the system was calculated from images of a 1000 line-pair-per-inch Ronchi reflective grating with the scanning mirrors operating at their maximum deflection angles. The maximum imaging depth of the system was determined in unlabelled, freshly excised normal human buccal mucosa using reflectance imaging, prior to the application of acetic acid. The maximum imaging depth was defined as the point at which the signal could not be distinguished from the background. The power throughput of the system was measured for all three illumination wavelengths. The visible 50/50 beam splitter was used for 488- and 664 nm illumination and the NIR 50/50 beam splitter for 784 nm illumination, with a reflective mirror as the sample, and the 20-µm-diameter pinhole in the optical path. The fluorescence detection limit of the system was determined by imaging dilutions of Alexa Fluor® 488 dye in solution (Invitrogen, Carlsbad, CA). Videos were taken of five samples of various concentrations of Alexa Fluor® 488 dye dissolved in phosphate-buffered saline (PBS), a sample of PBS only and no sample with the laser shuttered. The videos were taken with 488 nm excitation, 500-550 nm bandpass emission, a 100 µm-diameter pinhole in the optical path and a detector gain of  $1.4 \times 10^5$ . The detector gain, pinhole diameter and laser power settings were the same as those used for imaging fluorescently labelled human tissue slices, and were constant for all videos. Fifty frames from each video were analyzed. The average intensity of each frame was determined, using Image J software (National Institutes of Health), and the mean of these averages was calculated. The video taken of no sample with the laser shuttered was used to determine the dc offset of the system. The video of the PBS sample was used to measure the intensity of the background. The background (B) was defined as the mean intensity of the PBS frames minus the calculated dc offset. The videos of the samples of Alexa Fluor® 488 dye dissolved in PBS were used to calculate the signal intensity at various dye concentrations. The signal (S) for each dye concentration was defined as the mean intensity of the video frames minus the calculated dc offset. The signal-tobackground ratio was then calculated using S/B. The detection limit of the system was defined as the dye concentration where the calculated S/B was greater than or equal to two.

To further evaluate the DCM's imaging capabilities, images were acquired from cells, animal models and fresh tissue slices with and without fluorescence and reflectance contrast agents. The details of each of these experiments are discussed in the following sections.

## Cell culture

1483 head and neck squamous cell carcinoma cells (kindly provided by Reuben Lotan at the University of Texas M.D. Anderson Cancer Center, Houston, TX) yield a high expression of EGF receptors. Cells were cultured using standard tissue culture techniques with antibiotics in a 37°C humidified incubator. Details of the cell culturing techniques are discussed in Carlson (2006).

### Hamster cheek pouch model

Results from two male Golden Syrian hamsters, one experimental and one control, are reported in this study (Charles River Labs, Wilmington, MA). The experimental animal was treated with a 0.5% dimethylbenz[ $\alpha$ ]anthracene (DMBA) carcinogen in mineral oil three times per week for 8 weeks. The carcinogen was applied to the deepest recess of the right cheek pouch with a No. 5 camelhair brush. The control animal was treated only with mineral oil three times per week for the same duration. Prior to imaging, the animals were anesthetized with 5% pentobarbital sodium (Nembutal Sodium Solution, Abbott Laboratories, Chicago, IL). Animals were dosed at 90 mg/kg body weight delivered via intraperitoneal injection, and the cheek pouch was rinsed with saline solution to remove food debris. Supplements of 24 mg/kg pentobarbital sodium were administered every 30 min.

To expose the treated area, the cheek pouch was manually inverted by the handler over a specially designed platform that allowed the animal to lie in a recumbent position and contained a post over which the cheek pouch could be secured with an oring. The hamster was covered with a ThermaCare heat-wrap (The Procter & Gamble Company, Cincinnati, OH) to prevent hypothermia during prolonged anesthesia. After imaging, animals were euthanized with an overdose of pentobarbital sodium. The animal protocol was reviewed and approved by the M.D. Anderson Cancer Center and Rice University Animal Care and Use Committees. A small-animal veterinarian with extensive experience with the DMBA model of hamster cheek pouch carcinogenesis (L.G.C.) participated in all aspects of the study.

## Fresh tissue slices

Paired clinically normal and abnormal biopsies were obtained from a consenting patient at The University of Texas M.D. Anderson Cancer Center. After removal, the tissue was immediately placed in chilled phenol red–free culture media (Dulbecco's modified essential media, Invitrogen), and sectioned into approximately 250-µm-thick transverse slices using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL). The clinical protocols were approved by the Institutional Review Boards at The University of Texas M.D. Anderson Cancer Center, The University of Texas at Austin, and Rice University.

## Contrast agent labelling

*Cell culture.* The contrast agents used to label the cell culture were molecular-specific agents targeting the EGFR on the cell surface. The fluorescent contrast agent consisted of a biotin labelled primary anti-human EGFR antibody (Ab 10, clone 111.6, LabVision Corporation, Fremont, CA) and an Alexa Fluor<sup>®</sup> 488 streptavidin conjugate (Invitrogen). Cells were labelled using the procedure described in Hsu *et al.* (2004). The reflectance contrast agent consisted of 40-nm-diameter gold nanoparticles with anti-human EGFR antibodies (clone 29.1, Sigma-Aldrich, St. Louis, MO) absorbed on the surface. The gold conjugates were prepared following the method of Sokolov *et al.* (2003) and cells were labelled using the procedure discussed in Carlson (2006). After labelling, all cell samples were fixed in 10% buffered formalin.

*Hamster cheek pouch model.* In the hamster cheek pouch model of oral carcinogenesis, reflectance contrast was enhanced by a 5% acetic acid solution. The acetic acid solution was applied to the inverted hamster cheek pouch for 10 min using gauze soaked in the solution.

*Fresh tissue slices.* For the fresh tissue slices from the human biopsies, 6% acetic acid in  $1 \times PBS$  was used to enhance the

Sample	Instrument	λ <sub>ex</sub> (nm)	λ <sub>em</sub> (nm)	Power on Sample (mW)	Pinhole Diameter (µm)	PMT Gain
Alexa Fluor <sup>®</sup> dye Labeled Cells	DCM	488	500-550	0.20	100	$3.6 \times 10^4$
	Leica	488	500-550	0.0041	259	$3.6 \times 10^4$
Gold Labeled Cells	DCM	664	no emission filter	3.90	20	$2.4 \times 10^2$
	Leica	633	400-800*	0.053	49	$2.4  imes 10^2$
Hamster Cheek Pouch	DCM	664	no emission filter	5.70	50	$1.0 \times 10^3$
Fresh Tissue Slices acetic acid	DCM	784	no emission filter	7.80	20	$5.0 \times 10^{2}$
Fresh Tissue Slices Alexa Fluor® dye	DCM	488	500-550	0.20	100	$1.4\times 10^5$

 Table 1. Imaging parameters used for all samples imaged on the DCM and Leica confocal systems. \*Cannot image on Leica system without emission filter in optical path, so a large filter was chosen for reflectance imaging

reflectance contrast. This acetic acid solution has been shown to increase the backscattered signal from cell nuclei in *ex vivo* tissues (Collier *et al.*, 2000; Drezek *et al.*, 2000) and is often used *in vivo* to define abnormal areas of the cervix during colposcopy due to the whitening effect it has on abnormal tissue areas (Pogue *et al.*, 2000; Smith, 2000). A tissue slice was imaged immersed in the 6% acetic acid solution.

Similar to the fluorescent contrast agent used with the cultured cells, a molecular-specific fluorescent contrast agent was used to target EGF receptors in tissue. This contrast agent consisted of Alexa Fluor<sup>®</sup> 488 fluorescent dye conjugated directly to a primary anti-human EGFR antibody (clone 225, 1.8 mg/mL, Sigma-Aldrich) using a monoclonal antibody labelling kit (Invitrogen). A second tissue slice was labelled with the fluorescent contrast agent following the procedure described in Carlson (2006).

## Imaging

The microscope parameters used to image the samples are shown in Table 1. Cell samples were imaged with the DCM as well as the Leica SP2 AOBS confocal microscope available in the microscopy user facility at the University of Texas at Austin. Images were collected on the Leica system using a  $20 \times 0.70$  NA objective, and the imaging parameters on the Leica system were set to match those used on the DCM. This included matching the gains of the PMT detectors, using values provided by company representatives and published specifications, so that the supply voltages provided to each PMT resulted in the same gain. For example, to achieve a gain of 10<sup>4</sup>, the DCM PMT required a supply voltage of 500 V and the Leica PMT required 375 V. The pinhole diameters were set to the same Airy diameter based on calculations of the Airy disk radius for each system. Additional information required for the calculation of the Leica system Airy disk was provided by Leica customer service representatives. The laser power was measured at the objective back aperture for both systems, since this was the only location accessible

to measure power on the Leica system in the user facility. The power incident on the objective back aperture was set to the same value for each system. The power of the Leica system was then decreased relative to the power of the DCM to account for differences in frame rate, PMT quantum efficiency, light gathering capacity of the objectives and wavelengthdependent differences in scattering from gold nanoparticles. The values for frame rate and PMT quantum efficiency were provided by manufacturer specifications. The DCM frame rate was 19 times faster than the Leica frame rate; therefore, the power at the back aperture of the Leica was reduced by a factor of 19 to simulate a faster scanning speed. The ratio of the quantum efficiency of the Leica PMT at 488 nm, for example, to the DCM PMT was 2. Therefore, the power at the back aperture of the Leica objective was again reduced by a factor of 2 to account for this difference in quantum efficiency. The light gathering capacity of the objective was determined using two methods. For fluorescence imaging, where the fluorescence emission was isotropic, the comparison of the light gathering capacity was based on the objective's angle of acceptance, which was related to the objective's numerical aperture (NA). The light gathering capacity of the Leica objective (air-immersion, 0.70 NA) was a factor of 1.2 larger than the DCM objective (water-immersion, 0.80 NA). For reflectance imaging of gold nanoparticles, the comparison of the light gathering capacity was based on the percentage of the scattering phase function within the objective's angle of acceptance. The percentage of scattering within the objective's acceptance angle for 40-nm-diameter gold nanoparticles was 23.5% for the DCM at 664 nm and 33.5% for the Leica system at 633 nm. Therefore, the ratio of the light gathering capacity of the Leica to the DCM was 1.4. The power at the objective of the Leica system was reduced to account for these differences. Finally, the gold nanoparticle scattering efficiency difference, due to the difference in incident wavelength, was calculated using Mie theory for both systems, using 664 nm for the DCM and 633 nm for the Leica system. The scattering efficiency at 633 nm was a factor of 1.3 higher than at 664 nm, thus the

power at the back aperture of the Leica objective was again reduced to account for this difference.

Animals were imaged with the DCM in reflectance mode, before and after applying the acetic acid solution to look at the cellular morphology and architecture of the tissue. For each human biopsy, one of the fresh tissue slices was imaged in reflectance mode after the application of acetic acid. The other slice, labelled with the fluorescent contrast agent targeting EGFR, was imaged in fluorescence mode. Image tiling was performed for both slices so that image mosaics were captured covering the entire biopsy slice.

## Analysis of Cell Images

Ten successive images were acquired for each of the cell samples. The cell images were then analyzed to determine the average signal and signal-to-noise in each image. An equal number of cells were analyzed in the DCM and Leica images. The signal-to-noise ratio was determined from the standard deviation of the signal intensity over the 10 successive images.

## Results

#### System performance

The measured lateral resolution of the DCM ranged from 0.31 to 0.85  $\mu$ m depending on the wavelength of illumination and the pinhole diameter. The diameter of the imaged fluorescent microspheres ranged from 0.38  $\mu$ m with a 20- $\mu$ m-diameter pinhole to 0.52  $\mu$ m with the 200- $\mu$ m-diameter pinhole. The measured optical sectioning thickness ranged between 1.37 and 13.10  $\mu$ m. The individual lateral resolution and optical sectioning measurements made for each wavelength at each pinhole diameter are plotted in Fig. 3.

The FOV of the DCM can be changed by increasing or decreasing the voltage driving the scanning mirrors, which controls the deflection angle of the mirrors. The maximum FOV of this system, limited by the scan angles of the mirrors, was measured as  $300\times250~\mu m.$ 

The maximum imaging depth in reflectance mode was measured as approximately 250  $\mu$ m in fresh buccal mucosa; ranging from 240  $\mu$ m at 488 nm to 260  $\mu$ m at 784 nm. The imaging depth at 784 nm did not increase dramatically over the depth achieved at 488 nm, as might be expected, owing to the decreased sensitivity of the detector at 784 nm compared to 488 nm.

The power throughput of the system measured was very close to the theoretical values. At 488 nm, 1.7% of the light power from the laser reached the detector. Similarly, for 664 and 784 nm, the measured power transmissions were 2.2% and 1.0%, respectively. Power losses larger than expected occurred at the collimating lens L1 for all three wavelengths, and at the pinhole and lens L7 for 488- and 784-nm illumination.

The fluorescence detection limit of the system, defined as the dye concentration where the signal-to-background within an image was greater than or equal to 2 was 10 nM. The measured signal-to-background increased linearly with dye concentration, until the image saturated at 10- $\mu$ M concentration.

## Images of Cells

Figure 4 shows images of cells labelled with Alexa Fluor<sup>®</sup> 488 targeting EGFR on the cell membrane. Figure 4a was taken with the DCM and Fig. 4b was taken with the Leica confocal microscope with parameters matching those used on the DCM. The average intensity from the labelled regions in the DCM image was  $51.9 \pm 4.5$ . The average signal intensity in the Leica image was  $39.2 \pm 5.0$ . The average signal from the Leica image was within a factor of 1.5 of the DCM signal intensity. The signal-to-noise (S/N) measured in the DCM image was 43.3, and 62.4 in the Leica image. Figure 4c is an image taken on the Leica confocal microscope using more typical imaging parameters for that system. In this case, the average signal intensity in the image was  $75.0 \pm 9.7$ , which is greater than



Fig. 3. (a) Lateral resolution and (b) optical sectioning thickness as a function of the pinhole diameter for each illumination wavelength.



Fig. 4. Images of cells labelled with Alexa Fluor<sup>®</sup> 488 targeting the epidermal growth factor receptor. (a) DCM image, (b) Leica image taken with parameters matching the DCM parameters and (c) Leica image taken with more typical parameters for that system. Scale bars are 50 µm.



Fig. 5. Images of cells labelled with 40-nm-diameter gold nanoparticles targeting the epidermal growth factor receptor. (a) DCM image, (b) Leica image taken with parameters matching the DCM parameters and (c) Leica image taken with more typical parameters for that system. Scale bars are 50 µm.

the intensity of the signal in the DCM image, but the DCM was acquiring images 19 times faster than the Leica system.

Images of cells labelled with 40-nm-diameter gold nanoparticles targeting EGFR were acquired with both systems, with similar results. Figure 5a was taken with the DCM and Fig. 5b was taken with the Leica confocal microscope with matching parameters. The average signal intensity from the labelled regions in the DCM image was  $82.3 \pm 14.8$ . The average intensity in the Leica image was  $30.5 \pm 4.3$ . The average signal from the Leica image was within a factor of 2.7 of the DCM image and 46.8 in the Leica image. Figure 5c was taken on the Leica system using more typical imaging parameters for that system. In this case, the average signal intensity in the Leica image ( $82.2 \pm 6.7$ ) was the same as the intensity in the DCM image, but the DCM was acquiring images 19 times faster than the Leica system.

Overall, these images show that the DCM can acquire images with comparable signal intensity and S/N as a commercially available system with near-video-rate image acquisition.

## In vivo images of hamster cheek pouch

The hamster cheek pouch was imaged to demonstrate the DCM's ability to image an animal model of carcinogenesis *in vivo*. Reflectance imaging was used to look at the architecture and morphology of the epithelium in the hamster cheek pouch.

Figure 6a shows a reflectance image from a DMBA-treated hamster before the application of acetic acid. Figure 6b shows a similar region of tissue after the application of 5% acetic acid. Images of the exact same FOV were not obtained owing to movement of the tissue during the application of acetic acid. The cell nuclei are much brighter after applying acetic acid, making the high nuclear density easier to detect.

A reflectance video showing blood flow was acquired in a control hamster. The video was taken with the focal plane located approximately  $80 \,\mu\text{m}$  below the surface of the tissue. In normal, untreated hamster cheek pouch, the epithelial layer is only a few cell layers thick. In the video, shown in the supplementary video, leukocytes were observed rolling along the vessel wall.

## Ex Vivo images of human oral cavity tissue slices

Figure 7a shows an example of an image mosaic of a fresh transverse tissue slice from a tongue biopsy. This image was taken in reflectance mode after the application of a 6% acetic acid solution to brighten the cell nuclei. The mosaic contains  $9 \times 7$  frames covering a region of  $2.66 \times 1.71$  mm. This mosaic gives a complete picture of the structure of the tissue slice, showing both a histologically normal region of epithelium on the left with neighbouring moderately-differentiated invasive cancer on the right. The neighbouring cancer might have been missed without the ability to image the entire tissue



Fig. 6. Hamster cheek epithelium (a) before and (b) after the application of 5% acetic acid. Images are from an animal treated with DMBA for 8 weeks. Scale bars are 100  $\mu$ m.



Fig. 7. (a) Mosaic image of a transverse tissue slice from a tongue biopsy taken in reflectance mode after the application of a 6% acetic acid solution. The mosaic contains  $9 \times 7$  frames covering a region of  $2.66 \times 1.71$  mm. (b) Corresponding histology image. Scale bars are 1 mm.



Fig. 8. Reflectance (a, c) and fluorescence (b, d) images of transverse tissue slices of normal (a, b) and cancerous (c, d) tongue tissue taken after the application of acetic acid (a, c) and a fluorescent contrast agent targeting EGFR (b, d). Scale bars are  $100 \ \mu m$ .

slice. Figure 7b shows the corresponding histology image. In the histology image, the cancer is not detached from the neighbouring epithelium, as it is in the slice imaged with the DCM. In both images, the white line below the epithelium indicates the location of the basement membrane. Papillae in the epithelium can be seen in both images. Cell nests seen within the cancer region of the confocal image can also be seen in the histology image.

Images from paired normal and abnormal tissue slices are shown in Fig. 8 to demonstrate the DCM's ability to detect acetic acid-induced contrast of cell nuclei and the fluorescent contrast agent targeting EGFR with near-video-rate image acquisition. Figures 8a and c show reflectance images of transverse slices from normal and cancerous tongue tissue, respectively, taken after the application of acetic acid. The cancerous tissue was diagnosed as moderately-differentiated invasive cancer. The cell nuclei appear as white circular structures in these images. In the normal image (Fig. 8a), nuclei are very dense at the basement membrane, denoted by the white line, and spread apart as they move towards the surface of the tissue. This shows the stratified structure expected of normal oral mucosa. However, in the cancerous tissue (Fig. 8c), the density of nuclei is high throughout the entire tissue slice and a defined basement membrane is no longer present. An increase in the nuclear density and nuclearto-cytoplasmic ratio, as well as a loss of the stratified structure of the tissue, can be seen in the cancerous tissue compared to the normal tongue tissue. Figures 8b and d show fluorescence images of normal and cancerous tongue tissue, respectively, after labelling the tissue with the fluorescent contrast agent targeting EGFR on the cell membrane. Actively dividing cells will express EGFR. These cells are limited to the basal layer at the basement membrane in normal tissue. Therefore, EGFR labelling occurs only near the basement membrane in normal tissue, as shown in Fig. 8b. In cancerous tissue, all of the cells are rapidly dividing and express EGFR. As a result, labelling is seen throughout the cancerous tissue (Fig. 8d). Thus, an increase in the EGFR labelling can be seen that is related to the increase in EGFR expression in the cancerous tissue compared to the normal tissue.

### Discussion

The dual-mode confocal microscope was designed as a tool that has the capability to study multiple targets, including both endogenous and exogenous targets of interest, and to study contrast agent labelling and penetration in epithelial tissues. The DCM has the ability to image both reflective and fluorescent targets, and examples of cell lines and tissues labelled with Table 2. Comparison of the specifications of the dual-mode reflectance and fluorescence confocal microscope (DCM) and selected commercially available confocal systems. \*Values listed for the DCM are measured values. Values listed for the commercial systems were obtained from manufacturer specifications listed in product information brochures or obtained via company representatives. <sup>‡</sup>The resolutions for the Leica TCS SP2 AOBS system were calculated based on the objective numerical aperture (NA) given in the manufacturer specification. The lateral resolution was calculated using  $0.40^*\lambda$ /NA and the axial resolution using  $\lambda/(NA^2)$ . <sup>§</sup>Imaging depth given was listed as the working distance in the manufacturer specification. The imaging depth listed for the Leica TCS SP2 AOBS system is the objective working distance (from manufacturer specifications) minus the cover slip thickness that the objective is designed for.

System	Mode (Fluor/ Refl)	Wavelength (nm)	NA	Lateral Resolution (µm)*	Axial Resolution (µm)*	Maximum FOV (µm)*	Maximum Imaging Depth (µm)*	Acquisition Speed (fps)*
DCM	F & R	488	0.80	0.31	1.37	$300 \times 250$	250	15
		664		0.34	1.51			
		784		0.61	1.88			
Leica TCS SP2 AOBS	F & R	351	0.70			$750 \times 750$	$420^{\S}$	0.78
		364						
		458						
		476						
		488		$0.28^{\ddagger}$	$1.00^{\ddagger}$			
		496						
		514						
		543						
		594						
		633		0.36 <sup>‡</sup>	$1.29^{\ddagger}$			
Lucid VivaScope 2000	R	830	0.90	1	6-8	$475 \times 475$	150-500	30
							(depending on tissue type)	
Mauna Kea Cellvizio (HD-1800/80)	F	488	0.50	2.5	20	$240 \times 200$	80 <sup>§</sup>	12
Mauna Kea Cellvizio (S-1500)	F	488	0.42	3.5	15	$600 \times 500$	0 <sup>§</sup>	12
Mauna Kea Cellvizio (MiniO)	F	488	0.80	1.8	5	$240 \times 200$	30 <sup>§</sup>	12
Optiscan Five1	F	488	0.30	0.7	7	$475 \times 475$	250	1.66

Alexa Fluor<sup>®</sup> 488 dye, gold nanoparticles and weak acetic acid are shown. The DCM has a measured resolution limit of 0.31  $\mu$ m laterally and 1.37  $\mu$ m axially, with a maximum imaging depth of approximately 250  $\mu$ m in human mucosal tissue. The spatial resolution and signal-to-noise of the DCM are sufficient to permit imaging of sub-cellular structures and labelled targets within epithelial tissues. Also, the near-video-rate image acquisition and upright objective allow the system to be used to study cellular morphology and contrast agent labelling *in vivo* in animal models of cancer.

The performance of the DCM compares favourably to commercially available confocal systems. Table 2 shows a comparison of the specifications of the DCM and selected commercially available confocal systems. The Leica TCS SP2 AOBS is a tabletop confocal system. The configuration shown in Table 2 corresponds to the system that was used to image the labelled cells discussed previously. The Lucid VivaScope 2000 (Lucid, Inc., Rochester, NY) is the most similar system to the DCM in terms of the reflectance imaging capabilities of the systems. Both systems can acquire reflectance images using NIR wavelengths, although the Lucid system is set up

for reflectance imaging only. The Lucid system can image to a depth of 450 µm in the lip, 400 µm in bladder tissue, 350 µm in the skin and 250 µm in the tongue (Rajadhyaksha et al., 1999a). An imaging depth of 250 µm was obtained in the buccal mucosa using the DCM, which is similar to the skin and tongue imaged with the Lucid system. The image acquisition of the Lucid system is faster than the DCM, but the measured minimum lateral and axial resolutions of the DCM surpass that of the Lucid. The Lucid system is the only commercial system to have an image tiling function similar to that of the DCM. The Five1 (OptiScan Pty. Ltd., Victoria, Australia) is an endoscopic confocal system that uses a single optical fibre for illumination and detection, and physically scans the fibre to create a two-dimensional image. This system is designed for fluorescence imaging at 488-nm excitation. This system has the advantage over the DCM that it is fibre-based and can be used easily in vivo, but it acquires images nine times slower than the DCM so movement and breathing artefacts may be more of a problem in this system than the DCM. Finally, Mauna Kea Technologies (Paris, France) has also developed a line of fluorescence confocal fibre-based systems, the Cellvizio.

These systems have a similar acquisition speed but decreased resolution and more shallow imaging depth compared to the DCM.

Combining reflectance and fluorescence together in a single system allows for multi-modal imaging, such as acquiring images of fluorescently labelled cells with corresponding reflectance images providing structural information about the tissue; this enables researchers to place the labelled cells within a histologically meaningful context. The DCM described here has that capability. Two other dual-mode systems have recently been reported as well. Chou et al. designed a laser scanning module to fit most commercially available nonconfocal microscopes (Chou et al., 2005). The module produces images at three fps, has a FOV of 133  $\mu$ m using a 40× 0.75 NA objective and 84  $\mu$ m using a 63  $\times$  1.40 NA objective, and has an axial resolution limit of 0.63 µm (Chou et al., 2005). Li et al. modified a Lucid Technologies VivaScope 2000, adding a fluorescence channel at 488 nm to the system. The system has a frame rate of 10 fps, a FOV of  $440 \times 320 \ \mu m$  and a theoretical axial resolution of 1.20 and 0.66 µm at 830 and 488 nm, respectively (Li et al., 2005). Of all the commercial and non-commercial systems reviewed, this system most closely resembles the specifications and performance of the DCM.

The DCM described here has the capability to image in both reflectance and fluorescence modes with more than one wavelength. It has very high lateral resolution and optical sectioning for all three illumination wavelengths in the system. It has a FOV similar to most of the commercial systems, image mosaic capabilities and a maximum imaging depth comparable to clinical systems such as the Lucid VivaScope. It has real-time image acquisition and an upright objective so that *in vivo* animal imaging can be accomplished. It is not as compact and portable as systems such as the Lucid or Mauna Kea systems. However, with a few design modifications, it would be possible to make the DCM more compact and more easily portable. In addition to the ability to image both reflectance and fluorescence at multiple wavelengths with one single system, the DCM also has the advantage that it is much cheaper than the commercially available systems. The components used to construct the DCM cost less than \$50,000.

The dual-mode imaging capabilities of the DCM allow investigation of both morphological changes as well as molecular changes that occur in disease processes. For example, with one system we were able to investigate the changing nuclear density and nuclear-to-cytoplasmic ratio as well as the increased expression of the EGFR in neoplastic tissues. Single-mode systems would only be able to investigate one or the other of these factors. Analyzing both factors simultaneously will be advantageous when trying to detect and diagnose diseases in their early stages. The DCM's high resolution and near-video-rate image acquisition, and the growing inventory of molecular-specific contrast agents and disease-specific molecular markers holds significant promise for *in vivo* studies of disease processes such as carcinogenesis. Dual-mode confocal microscopy may also be useful for margin detection, sentinel lymph node mapping and studies of therapeutic agents, for which fluorophores may be used to probe the trafficking of specific cells or the therapeutic agents themselves.

This paper describes the design and performance of a nearvideo-rate dual-mode reflectance and fluorescence confocal microscope and its application for imaging carcinogenesis in ex vivo human tissues and in vivo animal models of the disease. Images acquired with the DCM demonstrate that the system has the sub-cellular resolution needed to visualize the morphological and molecular changes associated with carcinogenesis and has the capability to image animal models of disease in vivo. The DCM has the ability to image regions of interest much larger than a single FOV, and with the ability to image both reflectance and fluorescence, images of contrast agent labelling can be put into the appropriate histological context using corresponding reflectance images of the tissue. The DCM will be a valuable tool as we continue to develop non-invasive, in vivo imaging tools and research various contrast agent conjugations and delivery methods in an effort to increase the contrast between neoplastic and normal epithelial tissues.

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# **Supplementary Material**

The following supplementary material is available for this article:

Video Clip S1. Video showing blood flow in cheek pouch of control hamster. Leukocytes can be seen rolling along the vessel wall. Field of view is  $300 \times 250 \ \mu m$ .

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/10.1111/j.1365-2818.2007.01818.x (This link will take you to the article abstract)

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