Ralf Kiesslich and Thomas D. Wang, Section Editors

Vertical Cross-sectional Imaging of Colonic Dysplasia In Vivo With Multi-spectral Dual Axes Confocal Endomicroscopy

Zhen Qiu,[1] Supang Khondee,[2,3] Xiyu Duan,[1] Haijun Li,[2] Michael J. Mandella,[4] Bishnu P. Joshi,[2] Quan Zhou,[1] Scott R. Owens,[5] Katsuo Kurabayashi,[6,7] Kenn R. Oldham,[6] and Thomas D. Wang[1,2,6]

[1]Department of Biomedical Engineering and [2]Department of Internal Medicine, Division of Gastroenterology, [5]Department of Pathology, [6]Department of Mechanical Engineering, and [7]Department of Electrical Engineering, University of Michigan, Ann Arbor, Michigan; [3]Pharmaceutical Science Department, School of Pharmaceutical Sciences, University of Phayao, Thailand; [4]Department of Pediatrics, Stanford University School of Medicine, Stanford, California

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Pathologists evaluate histology sectioned perpendicular to the tissue surface, or in vertical cross-sections. This orientation (*X–Z* plane) enables evaluation of mucosal differentiation in the basilar-to-luminal direction. Current endomicroscopes use a conventional (single axis) optical design.[1] Imaging is limited to horizontal cross-sections (*X–Y* plane) where the microanatomy is frequently similar across the field of view (FOV). In the dual axes configuration, light is delivered and collected off-axis, and images can be detected over a much larger range of intensities.[2] Molecular images collected using fluorescence can improve specificity for disease detection and reveal functional properties about tissue.[3] Proper interpretation of these images requires correlation with the microanatomy. We aimed to demonstrate the simultaneous collection of 2 fluorescence images in vivo in vertical cross-sections using a dual axes confocal endomicroscope. An overlay of molecular and anatomic images from normal and dysplastic mouse colonic mucosa are displayed in real time.

Description of Technology

In the dual axes confocal endomicroscope, the illumination and collection beams travel along different paths, and the region of overlap defines the focal volume (4 μm lateral, 5 μm axial resolution)[4] (Figure 1*A*). A parabolic mirror weakly focuses the folded beams to create a long working distance. A tiny scan mirror (3×2 mm^2) is used to perform large deflections ($\pm 6^\circ$) at resonance (3.01 kHz) and achieve a large FOV (800 μm). A bulk piezoelectric actuator moves the scan mirror and hence focal point perpendicular to the tissue surface (400 μm depth) at 5 frames per second. Laser beams at $\lambda_{ex} = 671$ and 785 nm are delivered into the illumination fiber to excite a Cy5.5-labeled (AKPGYLS) peptide,[5] hereafter AKP*-Cy5.5, and IRDye 800, respectively. These fluorophores were chosen to minimize hemoglobin absorption and tissue scattering, reduce background from tissue autofluorescence, and

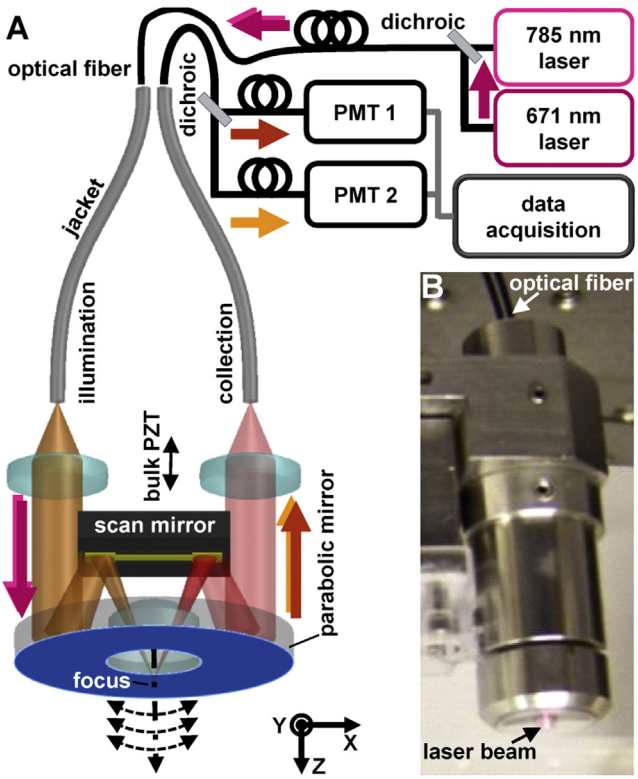

Figure 1. Dual axes confocal endomicroscope. (*A*) Schematic showing laser excitation at 671 and 785 nm delivered into the illumination fiber. The beams are focused by a parabolic mirror and delivered off-axis into the tissue, and the fluorescence is collected along the same path. A scan mirror deflects the beams in the *X–Y* plane, and a bulk piezoelectric actuator moves the focus perpendicular to the tissue surface. (*B*) Instrument.

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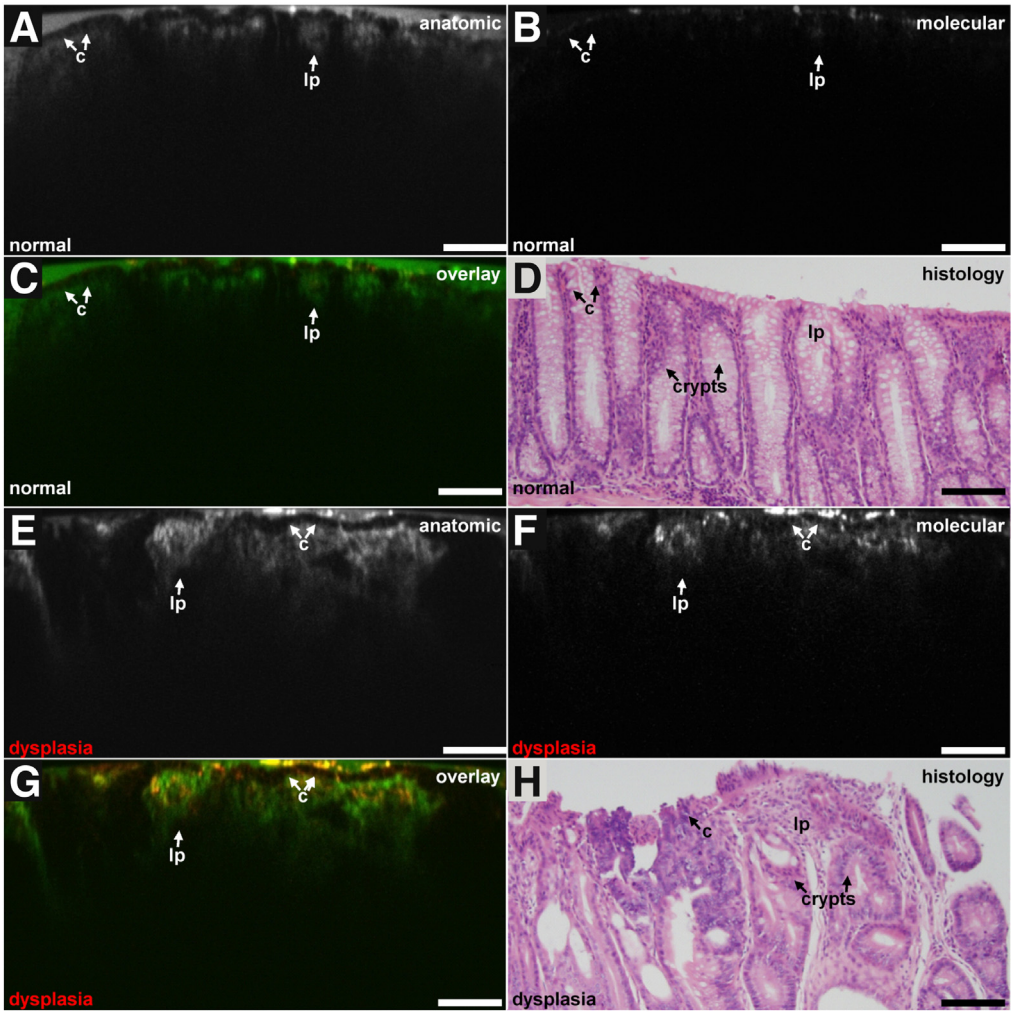

Figure 2. Vertical cross-sectional images. Anatomic, molecular, overlay, and histology images for *A–D* (normal) and *E–H* (dysplastic mouse colonic mucosa) are shown in vertical cross-sections. *Scale bar*, 100 μm.

provide maximum light penetration depth. Fluorescence is collected off-axis and travels along a symmetric optical path before being focused into a collection fiber. Fluorescence from the 2 fluorophores is separated by a dichroic mirror (740 nm) and detected by 2 separate photomultiplier tubes. The complete packaged instrument (10 mm OD) is shown (Figure 1*B*).

Mice that have been genetically engineered with a somatic inactivation of Apc to spontaneously develop adenomas in the distal colon were anesthetized with isofluorane (2% at 0.5 L/min).[6] The Cy5.5-labeled peptide (600 μmol/L, 200 μL) was injected via the tail vein. After 150 minutes to clear nonspecific binding, IRDye 800 (600 μmol/L, 200 μL) was then administered. The distal tip of the dual axes confocal endomicroscope was placed in contact with a prolapsed region of colonic mucosa, and ~2 mW of excitation at each wavelength was delivered into the tissues. Images from either fluorescence channel were recorded at the same photomultiplier tube gain. Video streams that showed minimum motion artifact, lack of debris (stool, mucus) covering the mucosal surface, and recognizable crypt morphology were identified.

Video Description

In the dual axes confocal design, the illumination and collection beams are separated and intersect at the focus below the tissue surface (Video Clip 1). In this geometry, subcellular resolution can be achieved, and very little of the light scattered by tissue along the illumination path is collected. Thus, light over a large range of intensities can be collected to produce vertical cross-sections. A long working distance is achieved using a parabolic mirror that weakly focuses the beams. The space created is used by a tiny scan mirror that deflects the beams horizontally, whereas a piezoelectric actuator moves the focus vertically.

The anatomic images (λ_{ex} = 785 nm, IRDye 800) from normal colonic mucosa (FOV 800 × 400 μm^2) reveal vertically oriented crypts with regular architecture and similar dimensions in height and width (Video Clip 2). A layer of water on the mucosal surface helps to couple the light into the tissue. The contrast agent seems to fill the lamina propria and surround individual colonocytes along the crypt periphery (Figure 2*A*). The fluorescence intensity decreases with tissue depth. The molecular images (λ_{ex} = 671 nm; AKP*-Cy5.5) show weak signal in the epithelium (background; Figure 2*B*). The

overlay images are shown in pseudocolor, and are dominated by the normal crypt anatomy (green) with minimal contributions from the molecular image (orange; Figure 2*C*). The corresponding histology over a similar FOV shows normal-appearing crypts lined by epithelium with colonocytes that have uniform, basally oriented nuclei (arrows) and abundant goblet-cells with mucin-filled vacuoles (Figure 2*D*).

The anatomic images from dysplastic colonic mucosa show irregular crypt architecture with variable dimensions and a complex branching pattern with an occasional cribriform arrangement (Figure 2*E*). The contrast agent seems to extravasate from the lamina propria. The molecular images show much greater fluorescence intensity around the surface of individual colonocytes (target; Figure 2*F*). The target-to-background ratio (average from 5 cells in each image) is 2.2. The overlay images show co-registration of peptide binding (orange) with colonocytes along the crypt periphery in the epithelium (Figure 2*G*). The corresponding histology shows dysplastic crypt epithelium with enlarged, hyperchromatic, and occasional vesicular nuclei that are no longer basally oriented (Figure 2*H*, *arrows*). Mucin production in the goblet cells is markedly decreased. An area of pronounced nuclear pseudostratification is apparent near the center.

Take Home Message

The dual axes confocal endomicroscope can collect vertical cross-sectional images over a large FOV (800 $\times$ 400 μm^2). Anatomic and molecular fluorescence images can be acquired simultaneously and co-registered in real time to identify the signal location. Images that reveal the molecular properties of tissues can be detected in vivo with the same orientation as that typically used by pathologists.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2014.01.016.

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Reprint requests
Address requests for reprints to: Thomas D. Wang, MD, PhD, Associate Professor of Medicine and Biomedical Engineering, Division of Gastroenterology, University of Michigan, 109 Zina Pitcher Pl. BSRB 1522, Ann Arbor, Michigan 48109-2200. e-mail: thomaswa@umich.edu.

Conflicts of interest
The authors disclose the following: The authors (ZQ, MJM, KK, KO, TDW) are inventors on patent applications filed on the instrument.

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