Microtome-Free 3-Dimensional Confocal Imaging Method for Visualization of Mouse Intestine With Subcellular-Level Resolution

YA–YUAN FU,* CHI–WEN LIN,‡ GRIGORI ENIKOLOPOV,§ ERIC SIBLEY,‖ ANN–SHYN CHIANG,‡ and SHIUE–CHENG TANG*

*Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan; ‡Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan; §Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; and ‖Division of Pediatric Gastroenterology, Stanford University School of Medicine, Stanford, California

BACKGROUND & AIMS: The intrinsic opacity of mouse intestinal tissue prevents its evaluation by high-resolution, in-depth optical microscopy. Instead, intestinal tissue is usually sectioned to expose the interior domains of the mucosa and submucosa for microscopic examination. However, microtome sectioning can cause distortions and artifacts that prevent acquisition of an accurate view of the sample. We therefore attempted to develop a microtome-free 3-dimensional (3D) confocal imaging method for characterization of mouse intestine. **METHODS:** We applied an optical-clearing solution, FocusClear, to permeate and reduce the opacity of mouse colon and ileum. Tissues were labeled with fluorescent probes and examined by confocal microscopy with efficient fluorescence excitation and emission in the FocusClear solution. The voxel-based confocal micrographs were processed with Amira software for 3D visualization and analysis. **RESULTS:** Treatment of tissues with the optical-clearing solution improved photon penetration, resulting in the acquisition of images with subcellular-level resolution across the mucosa, submucosa, and muscle layers. Collectively, the acquired image stacks were processed by projection algorithms for 3D analysis of the spatial relations in villi, crypts, and connective tissues. These imaging technologies allowed for identification of spatiotemporal changes in crypt morphology of colon tissues from mice with dextran sulfate sodium–induced colitis as well as detection of transgenic fluorescent proteins expressed in the colon and ileum. **CONCLUSIONS: This new optical method for penetrative imaging of mouse intestine does not require tissue sectioning and provides a useful tool for 3D presentation and analysis of diseased and transgenic intestine in an integrated fashion.**

To view this article's video abstract, go to the AGA's YouTube Channel.

Microscopic visualization of intestine under normal and diseased conditions is essential for understanding the physiology and developing screening and diagnostic tools for intestinal diseases.[1] Structurally, the intestine comprises the mucosa, submucosa, and muscle layers: a proliferating stem cell population located at or near the crypt base in the mucosa and undergoes terminal differentiation as cells migrate from the base to the luminal surface.[2] Because standard 2-dimensional (2D) tissue analysis confines visualization of the intestinal architecture at a specific cut plane, 3-dimensional (3D) representation of image data over an area of interest is preferable for in situ visualization and assessment of the epithelium.[3]

Among the available imaging technologies, confocal microscopy generates a sharp 2D image at the plane of focus; incrementing the plane of focus creates a series of optical sections at different depths in the specimen, which allows for construction of a 3D image.[4] When a tissue specimen is sufficiently transparent so that light can pass through with minimal scattering (such as the zebrafish embryo), confocal microscopy provides a useful tool to study the 3D configuration of molecules of interest in the sample. Unfortunately, tissues such as colon and small intestine are nontransparent, which seriously limits their optical accessibility for confocal microscopy or light microscopy in general.

To date, preparation of tissue sections has been the standard laboratory practice used to expose the interior domain of a thick tissue for microscopic exami-

Abbreviations used in this paper: 2D, 2-dimensional; 3D, 3-dimensional; DiD, 4-chlorobenzene sulfonate salt; DSS, dextran sulfate sodium; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PI, propidium iodide.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.05.008

nation. There are, however, practical difficulties in using thin layers of tissue sections to acquire an integral view of the sample: artifacts and distortions introduced by microtome slicing are inevitable, let alone the challenge of aligning serial sample sections with precision. Recently, significant progress has been made to develop the confocal laser endoscopy for in vivo diagnosis of gastrointestinal disorders.[5–9] However, the extension of confocal microscopy from the bench to the bedside still leaves unresolved the problem of light scattering as photons encounter the gut wall.

Previously, we have developed a set of bioimaging technologies in sample preparation, confocal microscopy, and postrecording image processing for visualization of neural circuits in the brain of fruit fly *Drosophila melanogaster* (~130 μm in thickness) at the subcellular level without using tissue dehydration, embedding, and sectioning.[10–13] In the present study, we extend the developed technologies for characterization of mouse intestine. We appreciate that the intrinsic opacity of the layers of mucosa, submucosa, and muscles can prevent efficient light penetration for high-resolution imaging. We therefore applied an optical-clearing solution (FocusClear, with a refractive index at 1.46; US Patent 6472216-B1) to permeate and reduce the opacity of the mouse intestine to improve photon penetration during optical microscopy.[14]

Unlike common optical-clearing procedures involving treatment with xylene, mineral oil, methyl salicylate, or glycerol, which often dissolve labeled fluorescent probes and result in weak signals and blurring images from the sample, the FocusClear-mediated opacity clearing is fully compatible with fluorochrome staining and fluorescent protein detection in the intestine. In this research, we show that the improved photon penetration led to a clear visualization of the mouse colon and ileum. To our knowledge, this is the first microscopic imaging method to achieve subcellular-level resolution (resolving adjacent nuclei) of the full depth of intestinal mucosa and submucosa without using microtome sectioning.

To demonstrate applications of this microtome-free confocal imaging method, we examined spatiotemporal changes in colonic crypt morphology after dextransulfate sodium (DSS)-induced ulcerative colitis in BALB/c mice.[15,16] We also inspected nestin-GFP (green fluorescent protein expression driven by the nestin promoter)[17] transgenic mice to show the 3D expression pattern of nestin in the colon and ileum. Results using optical clearing combined with fluorescence labeling and confocal microscopy for 3D visualization of mouse intestine are presented and discussed in this report.

Materials and Methods

Animals

Female 2-month-old wild-type BALB/c mice (18~20 g), obtained from BioLASCO Taiwan Co, Ltd (Taipei, Taiwan), were used as the normal control and subjects to induce ulcerative colitis. To develop colitis at different stages, the same batch of mice received 5% DSS (molecular weight [MW] 36,000–50,000 Da; MP Biomedicals Inc, Cleveland, OH) in the drinking water for 0 (the normal control), 3, 5, or 7 days before being killed for examination. Experiments were repeated thrice, and animals were examined each time to ensure the progression of the colitis. The nestin-GFP transgenic mice used in this research have been developed previously.[17] In these animals, the GFP expression is under the control of the 5.8-kb protomer and the 1.8-kb second intron of the nestin gene, which encodes a type VI intermediate filament protein. Overall, 10 nestin-GFP transgenic mice were investigated for their nestin expression pattern in the colon and ileum. The care of the animals was consistent with Guidelines for Animal Experiments, National Tsing Hua University, Taiwan.

Preparation of Specimens

A standard operating procedure for colon (distal) and ileum removal and flushing was followed. Cold Hank's balanced salt solution with 0.4 mol/L N-acetyl-L-cysteine was applied from both ends of the gut tube to remove luminal contents. The tube was then longitudinally slit open to expose the luminal surface and washed with the phosphate-buffered saline (PBS). Before dye staining, specimens were fixed with 4% paraformaldehyde and permeabilized with 2% Triton X-100. Specimens from the BALB/c mice were then stained with propidium iodide (PI; 62.5 μg/mL; Molecular Probes, Eugene, OR) for 30 minutes at room temperature to label the nuclei. After washing with PBS thrice, specimens were stained with DiD (4-chlorobenzene sulfonate salt; 2 μg/mL; Molecular Probes) overnight at room temperature to label the membranes. Specimens from the nestin-GFP mice were stained with PI only. Afterward, the labeled specimens were immersed in the FocusClear (CelExplorer, Hsinchu, Taiwan) solution for optical clearing before being imaged. The percentage of transmittance of light was measured by a microplate reader (SpectraMax M2e; Molecular Devices, Sunnyvale, CA) with specimens immersed in the optical-clearing reagents.

Imaging Settings

Confocal imaging was performed with a Zeiss LSM 510 confocal microscope equipped with a 40× or 40× LD "C-Apochromat" water immersion objective lens (Carl Zeiss, Jena, Germany). The PI- and DiD-labeled samples were excited with helium/neon lasers at 543 nm