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A microtome-free 3-dimensional confocal imaging method for visualization of mouse intestine with subcellular-level resolution

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Abstract

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 via projection algorithms for 3D analysis of the spatial relations in villi, crypts, and connective tissues. These imaging technologies allowed for identification of spatio-temporal 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.

Keywords

3D confocal imaging; 3D visualization; colitis; dextran sulfate sodium; nestin

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Introduction

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 is comprised of the mucosa, submucosa, and muscle layers: a proliferating stem cell population located at/near the crypt base in the mucosa and undergoes terminal differentiation as cells migrate from the base to the luminal surface.² Because standard two-dimensional (2D) tissue analysis confines visualization of the intestinal architecture at a specific cut plane, three-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 colons and small intestines are non-transparent, 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 examination. 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 post-recording image processing for visualization of neural circuits in the brain of fruitfly *Drosophila melanogaster* (~130 μm in thickness) at the subcellular level without employing 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 spatio-temporal changes in colonic crypt morphology following dextran sulfate sodium-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 reveal 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% dextran sulfate sodium (DSS, MW 36,000–50,000 Da, MP Biomedicals Inc., OH) in the drinking water for 0 (the normal control), 3, 5, or 7 days prior to being sacrificed 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 promoter and the 1.8-kb second intron of the nestin gene, which encodes a type VI intermediate filament protein. Overall ten 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 M 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). Prior to 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. Afterwards, the labeled specimens were immersed in the FocusClear™ (CelExplorer, Hsinchu, Taiwan) solution for optical clearing prior to being imaged. The percent transmittance of light was measured via 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 and 633 nm. A band-pass 560- to 615-nm filter and a long-pass 650-nm filter were used to collect signals from PI and DiD, respectively. Alternatively, a long-pass 650-nm filter was used to collect signals from both dyes and combine them to a single track. The PI-labeled specimens from the nestin-GFP mice were excited at 543 nm (PI excitation) and 488 nm (GFP excitation, an argon laser). A long-pass 560-nm filter and a band-pass 505- to 530-nm filter were used to collect signals from PI and GFP, respectively. Alternatively, we used the 543-nm laser as the light source and the transmitted light channel to examine specimens with the DSS-induced colitis.

Sample scanning was recorded every 0.21 µm (at X/Y resolution of 1024×1024 pixels) and the increment of the Z-axis optical section was 1 µm. The “line” scanning pattern was used and the scanning speed = 8. The pixel intensity, ranging from 0 to 255, was set to be the mean

value of four scans. Typically, it took 40~50 minutes for a full scanning process from the colonic luminal surface to the serosal surface, which generated 200 ± 10 optical slices, depending on the variation of the thickness. The size of the data was ~420 megabytes (1024×1024 pixels $\times$ 200 slices $\times$ 2 channels of signals) for a typical surface-to-surface scan.

Post-recording image processing and analysis

The voxel-based confocal micrographs were processed using the LSM 510 software (Version 3.2, Carl Zeiss) and Amira® 4.1.2 (Mercury, Chelmsford, MA) for orthogonal and stereo projections and analysis. It took less than one minute to upload the LSM image data into Amira®, which was operated under a Dell Precision T5400 workstation. Typically, Amira® responded to user commands in less than one minute. In Figures 4A–D, and Supplementary Video 1, the image stacks were displayed using the *Ortho Slice* function of Amira®. The video was made via the *Movie Maker* function of Amira® with the increase in display time in association with the depth of the optical section from the luminal surface (beginning) to the muscle layer (end). In Figures 4E, 5A–C, 6B–E, and 8A–F, the *Voltex* module of Amira® was used to project the image stacks and the *Trackball* function was used to adjust the projection angle. The intensity of confocal fluorescence was presented by the grayscale or pseudocolor scale bar(s).

Results

Optical clearing of the colon and ileum specimens

Gross images of mouse colons and ilea in view of the low-resolving power from a standard light microscope are shown in Figures 1A–D. The specimens are intrinsically opaque in saline (Figures 1A and C); thus, it is difficult to acquire images from the internal structure. Traditionally, intestinal tissues as well as other biological/non-biological samples are sectioned into thin slices to expose their interior domain for microscopic examination. However, the spatial information of the structure is often interrupted and/or lost during the sectioning process.

Previously, we have applied an optical-clearing solution, FocusClear™, to increase the transparency of the insect brains for mapping of neural circuits via confocal microscopy.^{10–14} We found that a similar clearing effect was observed when the FocusClear™ solution was applied to colon and ileum specimens (Figures 1B and D). Particularly, Figure 1B shows that when the mouse colon was immersed in the FocusClear™ solution, the whole tissue became transparent, leading to a clear visualization of the crypt openings at the luminal surface under the microscope.

We quantified the optical-clearing effect over a spectrum of wavelengths by measuring the percent transmittance of light across the ileum specimen. Figure 1E shows that the increase in transmission was associated with the increase in wavelength, owing to Rayleigh scattering.¹⁸ Specifically, when the wavelength was at 480~490 nm (a common range for excitation of green fluorophores), the FocusClear™ immersion promoted percent transmittance to 77% from 19% of the saline immersion (control), or by ~4-fold. Similar increases were also observed in other wavelengths longer than 400 nm. In addition, we measured the kinetics of optical clearing: Figure 1F shows that the transmission (at 488 nm) reached a plateau at 75% within 30 minutes of when specimens were immersed in the FocusClear™ solution.

Chemical reagents such as glycerol and dimethyl sulfoxide have been used to improve light penetration of gastric and skin tissues.^{19–21} Figures 1E and F, however, show that glycerol and dimethyl sulfoxide are less effective than the FocusClear™ solution in facilitating photon penetration across the ileum specimen.

Optical clearing enables deep-tissue confocal microscopy

Figures 2 and 3 compare confocal micrographs of mouse colon and ileum when specimens were immersed in saline (PBS, non-treated control) or FocusClear™ solution for penetrative imaging. For the non-treated colon specimen, the excitation laser and fluorescence emission were scattered from the epithelium, thus resulting in a drastic decrease in the signal-to-noise ratio over the depth. Particularly, resolving adjacent nuclei became difficult when depth = 20 μm and became unfeasible when depth = 40 μm (Figures 2C and E). In comparison, optical clearing led to acquisition of sharp confocal micrographs of colonic crypts at the same depths (Figures 2D and F).

Figure 3, on the other hand, compares confocal micrographs and the orthogonal projections of the non-treated and optical-cleared ileum specimens. Unlike the colonic crypts which curve into the luminal surface, the ileal villi protrudes; thus, the epithelial cells at the lining can be seen without optical clearing. However, the signal-to-noise ratio deteriorated when the fluorescence was from the interior domain of the ileum (Figures 3A, C, and E). This was caused by poor photon penetration along the Z-axis when the excitation laser and emission fluorescence traveled in the tissue. In comparison, optical clearing enabled a clear visualization of both the exterior and interior domains of the ileum specimen (Figures 3B, D and F). It is noteworthy that the image resolution of the X/Z and Y/Z planes of the orthogonal projection is lower than that of the X/Y plane. The lack of Z-axis resolution is an inherent limitation of the current optical and mechanical designs of confocal microscopy.

Penetrative 3D imaging of the mouse colon

Figures 4A–D are examples of the confocal micrographs at different depths of the distal colon: (A) The openings of colonic crypts at the mucosal surface, (B) the boundary of colonic crypts to the connective tissue (depth = 80 μm), (C) the blood vessels in the submucosa (depth = 123 μm), and (D) the muscle layer (depth = 150 μm). The additional high-resolution micrographs of the distal colon are presented in Supplementary Figure 1. Supplementary Video 1 presents serial optical sections from the luminal surface to depth = 150 μm . Unlike the image acquisition from the microtome sections, this approach is noninvasive and examines a region of mucosa and submucosa *in situ*, which provides a continuous flow of information along the crypt axis for microscopic inspection.

Taking advantage of voxel-based confocal micrographs, the acquired image stacks can be digitally processed to present the exterior and interior details of the scanned 3D region. Both Figures 4E and 4F were derived from the same set of the image stack shown in Figures 4A–D and Supplementary Video 1. Specifically, the capital letters “B,” “C,” and “D” on the Y/Z plane of Figure 4F indicate the locations of the images shown in Figures 4B, C, and D, respectively. Using these digital images, we can readily quantify the dimensions of colonic crypts in large numbers and *in situ*. Because there are no physical sections involved in sample preparation, the errors from distortions and artifacts can be minimized in measuring the crypt structure.

3D microscopy of the mouse ileum

Figure 5A displays the stereo projection of mouse ileum acquired from the fluorescent staining of cellular membranes and nuclei. The “luminal scan” and “serosal scan” shown in Figure 5B represent the use of the 3D optical method to visualize the luminal and serosal halves of the ileum. The two “serosal scans” at the middle and bottom parts of Figure 5B were projected from the same image stack, except that the bottom image has a cuboid digitally subtracted from the scanned region (via the volume-editing function of Amira®) to expose the interior domain of the crypt structure. The same volume-editing method was applied to Figures 5A and C.

Figure 5C shows a full-depth, 3D projection of the mouse ileum. Significantly, this is the first time that the microscopic ileal structure is presented in an integrated 3D fashion with subcellular-level resolution. The 360-degree panoramic presentations of Figures 5B (bottom) and 5C are shown in Supplementary Videos 2 and 3, respectively. These videos display the intricate villus and crypt arrays using multiple projection angles. In comparison, Figure 5D presents the standard 2-dimensional images at different depths of the ileum acquired from the luminal and serosal scans, which are a subset of images shown in Figure 5B.

3D confocal imaging of dextran sulfate sodium (DSS)-induced colitis

We next applied the 3D confocal imaging method to examine spatio-temporal changes of crypt morphology in colitis. We used oral administration of dextran sulfate sodium (DSS) to induce an acute colitis in mice. Seven days after ingesting 5% DSS in the drinking water, the recipient mice showed signs of bloody diarrhea, weight loss, and shortening of the colon (Figure 6A).^{16,17} It has been proposed that DSS is toxic to the colonic epithelial cells, and the loss of these cells compromises the integrity of the mucosal barrier to induce colitis. Previously, investigators were limited to the standard 2D tissue-section analysis, and could only characterize the DSS-induced colitis at a specific X/Y, Y/Z, or X/Z cut plane of the colon. For the first time, we applied confocal microscopy to noninvasively acquire optical slices across the diseased colon, from the luminal surface to the serosal surface, and used projection algorithms to generate panoramas of the scanned region for 3D characterization of colitis (Figures 6B–E and Supplementary Videos 4–7).

Figures 6B–E are projections of confocal micrographs that exhibit the progression of the DSS-induced colitis from day 0 (normal) to day 3, day 5, and day 7. Specifically, the left parts of Figures 6B–E are colon projections from the top of the luminal domain. These images show that the crypt openings gradually disappeared over time as the colitis progressed. Furthermore, we digitally subtracted a cuboid from the scanned region to expose the interior domain of the colon for inspection. The right parts of Figures 6B–E are stereo projections of the result. We used a major blood vessel at the boundary of mucosa and submucosa as the Z-axis reference for the volume subtraction, which marks the depth where the base of the crypt could reach. Noticeably, both at day 0 and day 3 the honeycomb-like network of the mucosa was visible, but at day 3 the ring-shape structure of epithelial cells was missing at the luminal surface. At day 5, the crypt structure was seriously deformed and only a portion of crypts could be identified. At day 7, the crypt structure disappeared, which accompanied an approximately 30% decrease in the colon length shown in Figure 6A.

The 360-degree panoramic presentations of Figures 6B–E are shown in Supplementary Videos 4–7, respectively. These videos present the change of the colonic crypt from different projection angles. These images and videos provide an entirely new approach, relative to the standard 2D tissue-section analysis, to present the spatial relations in crypt structure and colitis.

Application of transmitted light to characterize crypt structure in the DSS-induced colitis

Because the FocusClear™ solution greatly improved light penetration across the colon specimen, the colonic crypt structure can be readily visualized along the crypt axis without dye staining. Figure 7 characterizes the spatio-temporal change of colonic crypts in the progression of colitis using the transmitted light channel of confocal microscopy. This is similar to viewing cells in a standard microscope with an additional function so that the focal plane can be digitally adjusted to the depth of interest for imaging. Figures 7A–D are images of mouse colonic mucosa 10 μ m under the luminal surface. These images were taken 0, 3, 5, and 7 days after the DSS ingestion, where the progression of the induced colitis correlated well with the disintegration/disappearance of crypt structure. Specifically, at day 3 (Figure 7B), the colonic epithelial cells were largely missing at the top of the crypt structure, leaving the honeycomb-

like compartments empty at this level. This is contrary to the healthy mucosa at day 0 shown in Figure 7A, where colonic epithelial cells occupied the compartments and fully contacted with the lamina propria. Furthermore, when the focal plane followed the crypt axis and moved to depth = 50 μm , there resided epithelial cells but these cells aggregated and were not in contact with lamina propria (Figure 7E). At depth = 70 μm (Figure 7F), more spaces were filled by epithelial cells, and their aggregation and detachment from the lamina propria became less prominent.

Compared with the crypt destruction at days 5 and 7 after the DSS ingestion, the change of crypt structure at day 3 was modest; yet, our imaging data (Supplementary Video 5 and Figures 6C, 7B, 7E, and 7F) clearly indicate that the toxicity of DSS to the colonic epithelial cells started as early as day 3, as evidenced by the decrease in crypt height of the treated epithelium. Previously, Tessner *et al.* showed that DSS induces a decrease in the number of proliferating cells per cecal crypt in the treated C3H mice.²² Because the life-span of colonic epithelial cells is 3–5 days, the lack of the proliferating stem/progenitor cells to renew the DSS-treated epithelium depleted the crypt structure at and close to the mucosal surface. This left the lamina propria alone in structuring the honeycomb-like mucosa without the epithelial cells filling at the top of the crypt (Figures 6C and 7B).

3D confocal imaging of transgenic mice carrying fluorescent proteins in the intestine

Transgenic mice carrying marker proteins have been widely used in studying gut development, physiology, and diseases. Mapping the 3D expression pattern of the fluorescent protein in transgenic mice provides a new way to analyze the arrangement of labeled cells or structures of interest in an integrated fashion. Figure 8 and Supplementary Videos 8–12 are the result of using the developed 3D confocal imaging method to map the nestin-GFP expression in the transgenic mouse colon and ileum. In these transgenic animals, the GFP expression is under the control of the promoter and the second intron of the nestin gene, which encodes a type VI intermediate filament protein. These nestin-GFP mice were originally developed to visualize neural progenitor cells and neuroepithelial stem cells in the brain.¹⁷ Later, the nestin-controlled GFP expression was found useful in imaging the capillary blood vessels in the skin and human tumor xenografts, including the colorectal cancer tissue.^{23–25}

Supplementary Video 8 displays the serial optical sections of the colon, from the luminal surface to the serosal surface, of the nestin-GFP mouse. Stereo projections of the image stacks from the luminal domain are shown in Figure 8A and Supplementary Video 9, which exhibit a unique pattern: the crypt arrays are surrounded by the nestin-GFP expression network in the mucosa. Figures 8B and C are the 3D penetrative projections of the nestin-GFP expression network, which resembles the capillary network in the lamina propria.²⁶ Figure 8D and Supplementary Video 10 are projections of the confocal image stacks from the serosal domain. A cuboid was subtracted from the scanned volume to reveal the interface between the bottom of the crypts and submucosa, where the nestin-GFP expression follows the path of blood vessels that circulate the crypts. Using the 3D image data, Supplementary Video 11 provides a fly-through presentation of the nestin-GFP colon. In addition, we mapped the 3D expression pattern of nestin-GFP in the ileum (Figures 8E and F and Supplementary Video 12), which also revealed the capillary-like network in the mucosa. Overall, the 3D figures and videos shown in this report represent data of confocal micrographs in a 3D space, and make possible viewing the spatial relations of intestinal tissues, which otherwise cannot be easily portrayed via the 2D tissue-section analysis.

Discussion

The intestinal epithelium possesses a fast proliferating stem cell population and a spatial pattern of terminal cell differentiation. It is a challenging task to use the standard 2D analysis of tissues

-- based on the information from microtome sections -- to provide an integrated view of the gene expression and cellular networks. In this research, we overcome the optical limitation and develop a microtome-free 3D confocal imaging method for characterization of mouse colon and ileum in depth. We connected three critical features of applying confocal microscopy for 3D tissue imaging and analysis: opacity clearing, fluorescent probe labeling, and post-recording image processing and projection. Together, we achieved high-resolution imaging of the colon and ileum specimens, which allowed us to observe spatial relations among the fluorescence-labeled structures. We provide an example of examining spatio-temporal changes in colonic crypt morphology following the DSS-induced ulcerative colitis. In another example, we revealed the nestin expression network in the colon and ileum using GFP as the transgenic marker. Both examples provide 3D perspective views of the specimens with subcellular-level resolution.

FocusClear™ is an aqueous solution selected from a group of chemicals consisting of sugars and their derivatives with high refractive indices (US Patent 6472216-B1). The application of the solution reduces the amount of refractive mismatch between tissue constitutes (high refractive index) and fluids (low refractive index), therefore avoiding random scattering and making the intestinal tissue transparent to facilitate laser penetration and fluorescence detection in confocal microscopy.

Because our specimen preparation is compatible with the routine histological processing procedure, a direct transfer of this new imaging approach to clinics would be in helping pathologists examine intestinal biopsies. The conventional microtome-based optical microscopy of patients' specimens commonly produces diagnostic errors largely due to artifacts, incomplete sampling, or poor orientation of embedded specimens.³ Our microtome-free 3D confocal imaging method provides a solution to these problems. However, it should be noted that prior to dye staining, we did adopt a gentle fixation and permeabilization protocol in specimen preparation. Although this step preserved the tissue structure and improved dye diffusion, studies comparing the freshly excised and the formalin-treated colorectal specimens have shown tissue shrinkage after fixation.²⁷ Thus, although our microtome-free imaging method avoids the distortion and artifacts introduced by microtome slicing, there are still disparate observations in dimensions of colonic structure between *in vivo* and *in vitro* measurements.

Due to light scattering and absorption in biological tissues, the laser beams and the fluorescence emission are unable to pass through tissue more than 100 μm thick in general.²⁸ Although recent advances in two-photon/multi-photon microscopy coupled with infrared lasers improves laser penetration and thus excites fluorescent probes twice deeper in comparison to conventional confocal microscopy, the resolution is not significantly improved because the emitted signals still need to travel through the opaque tissue for detection. In this research, the FocusClear™-aided optical clearing of intestinal specimens improves the tissue transparency (Figures 1B and D). The substantial increase in photon penetration allows not only efficient excitation/detection of fluorescent signals but also the use of transmitted light as an effective means for crypt visualization (Figure 7). Because no fluorescent staining is required in the later case, it provides a straightforward method for a quick inspection of the colon epithelium.

In summary, this research constitutes an important step toward developing an optical microscopic method for mapping the 3D structure of the digestive tract. The integration of FocusClear™-mediated optical clearing with confocal microscopy provides an opportunity to target tissues such as the colon and ileum for high-resolution image acquisition without microtome sectioning. Future work will be aimed at extending the new tool for imaging the stomach and pancreas, as well as analyzing of intestinal diseases, physiology, and gene expression in an integrated 3D fashion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Optical clearing of mouse colon and ileum specimens

(A and B): colon. (C and D): ileum. The specimens were immersed in phosphate-buffered saline (A and C) or FocusClear™ solution (B and D) prior to being imaged by a standard light microscope (Nikon TS100). Bar = 100 μ m. (E) Percent transmittance of light across the ileum over a spectrum of wavelengths, from 300 to 750 nm. (F) The kinetics of optical clearing (applied wavelength: 488 nm). In (E) and (F), black, green, blue, and red lines represent specimens immersed in saline, dimethyl sulfoxide, glycerol, and FocusClear™ solution, respectively. Results are presented as mean $\pm$ standard deviation (n = 5).

Figure 2. Comparison of confocal micrographs of mouse colon when specimens were immersed in saline or FocusClear™ solution

Cell nuclei were revealed by PI staining. (A, C, and E): saline immersion. (B, D, and F): FocusClear™ immersion. (A and B), (C and D), and (E and F) were acquired at the luminal surface and 20 and 40 μ m under the surface, respectively. Bar = 50 μ m. In order to detect the nuclei, the detector gain was adjusted from 36% (surface) to 57% (Z: 40 μ m) and from 33% (surface) to 38% (Z: 40 μ m) while imaging the non-treated and optical-cleared specimens, respectively. Similar adjustments were applied to other specimens to compensate the decline in confocal fluorescence over the imaging depth.

Mouse ileum
(control specimen, saline immersion)
X/Y plane at Z = 170 μm under the villus tip

Detector gain = 58%

Mouse ileum
(optical-cleared specimen, FocusClear™)
X/Y plane at Z = 170 μm under the villus tip

Detector gain = 40%

X/Y plane at Z = 320 μm

X/Y plane at Z = 320 μm
(close to crypt tips)

Figure 3. Comparison of confocal micrographs and orthogonal projections of mouse ileum when specimens were immersed in saline or FocusClear™ solution
 Cell nuclei were revealed by PI staining. **(A, C, and E)**: saline immersion. **(B, D, and F)**: FocusClear™ immersion. **(A and B)** were acquired at 170 μm under the villus tip. **(C and D)** and **(E and F)** were the orthogonal projection and the orientation of the X/Y, X/Z, and Y/Z planes in 3D space (using the *Ortho Slice* function of Amira®). The green lines in **(C and D)** indicate the boundaries of the X/Y, X/Z, and Y/Z planes shown in **(E and F)**. Dimensions of the scanned volume: 210 μm (X) $\times$ 210 μm (Y) $\times$ 380 μm (Z, depth).

Figure 4. Penetrative confocal imaging of mouse colon

(A–D) Confocal micrographs at different depths, starting from the luminal surface (A), the boundary of colonic crypts to the connective tissue (B), the blood vessels in the submucosa (C), to the muscle layer (D). Cells in the specimen were stained by PI and DiD to reveal the nuclei (bright spots) and membranes. Bar = 50 μm. (E) Stereo projection of the colon image stack creates a 3D visualization of the exterior part of the imaged region. (F) Orthogonal projection of the image stack reveals the interior configuration of the imaged region. Colonic crypts can be identified on the X/Z and Y/Z planes.

Figure 5. 3D microscopy of mouse ileum

(A) Stereo projection of confocal micrographs acquired from the DiD-stained membranes (gray) and PI-stained nuclei (orange). (B) Stereo projection of the confocal “luminal scan” and “serosal scan” to visualize the luminal and serosal halves of the ileum. Arrows indicate the scan directions. (C) A full-depth, 3D projection of the ileum. (D) Standard 2-dimensional images of the villi, crypts, tips of crypts, and the muscle layer. Images were extracted from the overall scanned regions shown in (B). The depths were indicated by the paired symbols “*,” “†,” “‡,” and “•” in (B) and (D). Dimensions of the scanned volumes: $210\ \mu\text{m}$ (X) $\times$ $210\ \mu\text{m}$ (Y) $\times$ $155\ \mu\text{m}$ (Z, depth) for (A) and (B (luminal scan)), $210\ \mu\text{m}$ (X) $\times$ $210\ \mu\text{m}$ (Y) $\times$ $145\ \mu\text{m}$ (Z) for (B (serosal scan)), and $290\ \mu\text{m}$ (X) $\times$ $290\ \mu\text{m}$ (Y) $\times$ $345\ \mu\text{m}$ (Z) for (C).

Figure 6. 3D Visualization of DSS-induced colitis

(A) Gross image of the DSS-treated mouse colon over a period of seven days. A significant ~30% shortening of the colon was found at day 7. (B–E) are projections of the distal colon at day 0 (normal), day 3, day 5, and day 7 post DSS ingestion. The left parts of (B–E) are projections from the top of the luminal surface. The right parts of (B–E) are stereo projections with a cuboid subtracted from the scanned volume to expose the interior domain of the colonic structure. Dimensions of the scanned volume: 210 μm (X) $\times$ 210 μm (Y) $\times$ 200 μm (Z). The 360-degree panoramic presentations of (B–E) are shown in Supplementary Videos 4–7, respectively.

Figure 7. Characterization of DSS-induced colitis using the transmitted light channel of confocal microscopy

(**A–D**) are representative micrographs of the distal colon at day 0 (normal), day 3, day 5, and day 7 post DSS ingestion. Images were taken 10 μm under the luminal surface. (**E and F**) were taken 50 μm and 70 μm under the luminal surface at day 3 post DSS ingestion. Dimensions of the scanned surface: 210 μm $\times$ 210 μm .

Figure 8. 3D visualization of the nestin-GFP expression networks

(A) 3D projection of the nestin-GFP expression in the colonic mucosa. Nuclei were revealed by PI staining (red). (B) Penetrative projection of the nestin-GFP expression networks shown in (A) after removing the signals from nuclei. (C) Penetrative projection of the nestin-GFP expression networks from the top of the luminal surface showing the honeycomb-like structure. (D) 3D projection of the nestin-GFP expression from the serosal domain. A cuboid was subtracted from the scanned volume to reveal the interface between the bottom of the crypts and submucosa. Dimensions of the scanned volume: 210 μm (X) $\times$ 210 μm (Y) $\times$ 200 μm (Z). The serial optical sections used to generate the projection are shown in Supplementary Video 8. The 360-degree panoramic presentations of (A and D) and a fly-through presentation of (D) are shown in Supplementary Videos 9–11, respectively. (E and F) 3D projection of the nestin-GFP expression networks in the ileal mucosa. (E) Signals of nuclei cover the nestin-GFP networks. (F) Signals of nuclei were digitally subtracted at the top corner to reveal the capillary-like network of nestin-GFP. Dimensions of the scanned volume: 230 μm (X) $\times$ 230 μm (Y) $\times$ 255 μm (Z). The 360-degree panoramic presentation of (F) is shown in Supplementary Video 12.