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3-D illustration of network orientations of interstitial cells of Cajal subgroups in human colon as revealed by deep-tissue imaging with optical clearing

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In the colon as well as other parts of the digestive tract, interstitial cells of Cajal (ICC) integrate with nerves and muscles as parts of the machinery to generate and regulate motility (16, 20, 22, 32, 41). The colons of most mammals studied generate slow waves and myenteric potential oscillations to modulate colonic motor function (17, 24, 30, 33–35). The two electrical depolarization patterns are thought to originate from the networks of ICC at the interface between the submucosa and circular muscle (slow waves) and around the myenteric plexus, respectively. During the past two decades, the understanding of ICC in health and disease (such as constipation, diabetes, and mutations) (3, 8–10, 12, 29, 40, 43) has led to the recognition of the role of ICC in motility disorders and the concept of studying the morphological changes of ICC to better characterize them (18, 19, 36).

Currently, the standard method to identify the gastrointestinal ICC is by immunostaining of the receptor tyrosine kinase c-kit (1, 15, 27). The c-kit signaling pathway is essential for the development and function of ICC. Morphologically, ICC are defined as the c-kit-positive cells with at least two processes extending from the cell body (this definition excludes the mast cells, which are also c-kit positive but do not have cellular processes) (3). With this approach, investigators have identified ICC subgroups at different gut locations and their association with nerves and muscles (4, 14, 23, 39).

However, the opaque nature of the gut presents a significant barrier to adequate observation of ICC networks in a three-dimensional (3-D) space continuum. For example, researchers routinely use microtome sectioning to improve photon penetration across the specimen (typically 10 μm or less in thickness) to detect the slender processes of ICC (which are around unit μm in width and can reach 100–200 μm in length) in microscopy (18). Although experienced investigators can conceive a virtual network based on the two-dimensional (2-D) images, the field has not been able to image ICC in depth, at the level of hundreds of micrometers, to provide a readily visualizable global view of the ICC network. It should also be noted that although previous studies have described 3-D imaging of ICC in whole mounts or thick tissue sections (e.g., at ~100 μm) (13, 21, 26, 28, 37), the earlier work did not address the problem of light scattering causing a decrease in image resolution or loss of signals along the focal depth in the tissue (25). Particularly, the depth limitation is a critical factor in verifying...
the 3-D features of the varicose processes of ICC and their connections in space.

To overcome these hurdles, we have applied confocal microscopy with optical clearing to visualize mouse and human intestinal structures (5–7, 25). Optical clearing is a technique that uses a solution of high-refractive index to match that of the tissue constituents to reduce light scattering, thus facilitating photon penetration in optical imaging (2, 11, 38). In this study, we integrate c-kit immunostaining with optical clearing and image rendering via 3-D microscopy to present the location-dependent network architectures of ICC in the tunica muscularis of the human colon with subcellular-level resolution (by resolving adjacent nuclei). Our 3-D illustration of ICC around the myenteric plexus (ICC-MY), in the longitudinal (ICC-LM) and circular (ICC-CM) muscles, and at the submucosal border (ICC-SM) provide multiple projection angles to identify the orientations of the ICC plexuses. In addition, this technique also allows us to reveal septal ICC that are reported to conduct pacemaker activity into the circular muscle (22, 23). The development of this new ICC imaging approach and its application to geometric illustration and quantitation of ICC are presented and discussed in this report.

**MATERIALS AND METHODS**

**Human specimens.** Collection and use of human tissues were approved by the Institutional Review Board of National Taiwan University Hospital—Hsinchu Branch and written consent from the patients to use their tissues was obtained. Colonic tissues were obtained from colectomies carried out for nonobstructing carcinoma. Tissue samples of the different ICC subgroups revealed in this paper. Table 1 lists the gender, age, and location of the sampled colon segments of subjects 1–4. Figures 1–7 are derived from four subjects. Figures 8 and 9 and Figs. 2–6, the Voltex module was used to project the 3-D function of Avizo to present the ICC plexus in Fig. 5. In Fig. 1, the volume editing function was used to subtract a cuboid from the scanned volume to reveal the ICC-LM layers for visualization. The camera rotation function was used to create the 360-degree panoramic displays of the 3-D images. Image segmentation and feature extraction were performed by the label field function of Avizo to present the ICC plexus in Fig. 5. In Fig. 1, D and F, the profile analysis module of the Zen software was used to reveal the change of the signal intensity along the pixel line at the center of the micrograph. Figures 7B, 8, 9B, 9D, and 9F were derived from the 3-D projection module of the LSM 510 software.

**RESULTS**

**Deep-tissue microscopy of ICC with optical clearing.** The human colon wall strongly scatters light (Fig. 1A). We reduced the scattering by immersing the colon wall in the optical-clearing solution of high-refractive index, similar to that of the tissue constituents, to promote photon penetration. The optically cleared specimen allows investigators to directly see the microstructures by transmitted light microscopy (Fig. 1B). The clearing process also reveals the tissue boundaries in the tunica muscularis of the human colon with subcellular-level resolution (by resolving adjacent nuclei). The average density derived from the 4 subjects is 2,512 cells/mm³. There was no statistical difference between any 2 subjects (P > 0.05 in all cases).
Fig. 1. Optical clearing of the human colon wall and c-kit immunostaining. A and B: optical clearing promotes photon penetration across the specimen of the human colon wall. The microstructures and their associated interstitial cells of Cajal (ICC) subgroups are listed in the figure. MY, myenteric plexus; LM, longitudinal muscles; CM circular muscles; SM, submucosal boundary. Thickness: 400 µm. C–F: immunostaining acquired from 2 sources of c-kit antibody. C and D: company MBL; E and F: company Epitomics. The vertical pixel line in the middle of D (1,024 x 1,024 pixels) correlates to the vertical axis of the signal intensity panel (right), which indicates the signal profile of the pixel line (25). D and F follow the same arrangement. Between the 2 sources, Epitomics’ antibody generates less background noises, leading to higher signal-to-noise ratios (i.e., higher image quality) to identify the processes of ICC in the projection (E).
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muscularis (submucosa, circular muscle, myenteric plexus, and longitudinal muscle) to select specific views of ICC subgroups.

Among the variables in immunostaining, the performance of the c-kit antibody is the key factor that affects the image quality to identify ICC. Figure 1, C–F compares the results of c-kit immunostaining using antibodies from two sources. As can be seen, the c-kit antibody from company MBL (Fig. 1, C and D) generates significant background noises, possibly due to nonspecific binding, that is seen both in the images and the profile analysis of the fluorescence signals. In comparison, noise is less prominent in the immunostaining performed by the antibody from Epitomics (Fig. 1, E and F). The critical difference between the two antibodies appears to be that the former nonspecifically stains the myenteric ganglia (Fig. 1, C and D). Importantly, the increased signal-to-noise ratio with the latter antibody in Fig. 1, E and F allows the slender processes of ICC to be distinguished, which is essential for ICC identification.

Fig. 3. ICC-LM in the longitudinal muscle. A: optical clearing reveals the texture of the longitudinal muscle. Arrows indicate two interfaces between the muscle segments, which correlate to 2 of the ICC-LM strata shown in E. B: representative 2-D micrograph of the c-kit (red) and nuclear (green) signals in the longitudinal muscle. Arrows indicate the cell bodies of ICC. The serial optical sections of the scanned volume are shown in Supplemental Video 3. C and D: merged and individual 3-D projections of the ICC-LM (gray) and nuclei (green) shown in Supplemental Video 3. E and F: volume-edited projections of c-kit signals reveal the orientations of the ICC-LM. A cuboid of the c-kit projection was digitally subtracted from the center of the scanned volume to expose the orientations of the ICC-LM strata which line the boundaries of the muscle segments (arrows in A) and extend toward the serosal and the longitudinal directions. A 360-degree rotation of the projection is shown in Supplemental Video 4.

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In the next four sections we show the results of c-kit immunostaining using the second antibody to reveal the networks of ICC subgroups as indicated in Fig. 1B.

**Periganglionic ICC-MY joint with ICC-LM and ICC-CM.** We first examined ICC-MY to verify the acquired c-kit signals in space. We chose ICC-MY because of their recognizable morphology around the myenteric ganglia (Fig. 2A). As can be seen, optical clearing reveals the different texture patterns of circular and longitudinal muscles, facilitating the identification of periganglionic ICC-MY at the myenteric plexus. However, unlike the conventional 2-D image in a specific plane of cut, serial micrographs along the focal path (depth: 200 μm; 81 optical sections, 2.5 μm per increment) provide a continuous flow of anatomic information (Supplemental Video 1). Figure 2, B and C are the merged and individual projections of the c-kit and nuclear signals shown in Supplemental Video 1. We projected the ICC images from different angles to generate panoramic views of the network structure (Fig. 2, C and D). Two more examples are presented in Fig. 2, E and F. Supplemental video 2 shows a 360-degree panoramic view of 2E. Morphologically, these in-depth 3-D images of high resolution also show the connections between subsets of the periganglionic ICC-MY and the ICC in the longitudinal (ICC-LM) and circular (ICC-CM) muscles.

**ICC-LM within the longitudinal muscle.** Figure 3, A and B show the optically cleared colon longitudinal muscle and a 2-D image of ICC-LM under the same view. The transparent longitudinal muscle specimen allows for penetrative 3-D microscopy to visualize the network orientation of ICC-LM. Figure 3, C and D is the merged and individual projections of the c-kit and nuclear signals (the source signals are shown in Supplemental Video 3). Importantly, ICC-LM appear to be organized in strata and their orientations were revealed when we rotated the c-kit projection. Figure 3, E and F shows the orientations of four of these strata of ICC-LM from two projection angles. A 360-degree panoramic view of the pro-
jection is shown in Supplemental Video 4. Taken together, the 3-D images and the muscle texture shown in Fig. 3A reveal the ICC-LM strata within the longitudinal muscle, extending toward the serosa.

3-D orientations of the repetitive and organized ICC-CM network. Figure 4, A and B shows the merged and individual projections of the c-kit and nuclear signals in the transverse colon section. Projections from this point of view show the bipolar ICC cells running parallel to the circular muscle axis, as indicated by the orientation of the nuclei. Importantly, when the projection rotates 90 degrees along the radial direction, the ICC-CM are also seen in strata and aligned in an organized manner from the top of the myenteric plexus toward the submucosa (Fig. 4C). A 360-degree panoramic view of this organization is presented in Supplemental Video 5.

The strata of ICC-CM can also be seen in the longitudinal colon section. Figure 4, D and E shows the circular muscles and the sandwiched ICC-CM in between. The staining of α-smooth muscle actin and c-kit reveals that the actin fibrils and ICC-CM are in close contact and lay down nearly parallel to each other (Fig. 4F and Supplemental Video 6), showing their intimate relationship.

To visualize a single layer of ICC-CM, we employed image segmentation to extract the c-kit signals and traced their paths across the scanned volume (Fig. 5 and Supplemental Video 7). Specifically, the nuclear signals indicate the cell bodies, confirming their ICC nature: connection of c-kit+ cells with at least two processes extending from the cell body. The definition is critical in quantitative analysis of ICC and will be discussed at the end of the results section.

ICC-SM layer lining the submucosal boundary. The optically cleared colon wall reveals the boundary between the circular muscle and submucosa in which a dense layer of ICC-SM resides (Fig. 6A). Typically, only a portion of the ICC cell body or processes can be seen by the conventional 2-D tissue analysis with a thin section at ~10 μm. We applied 3-D microscopy to map the ICC-SM over a depth of 300 μm to trace the plexus along the submucosal border (Supplemental Video 8). Rotation and signal segmentation of the image stack (Figs. 6, B–F and Supplemental Video 9) reveal that ICC-SM line the interface of circular muscle and submucosa, following its curvature in space. This arrangement resembles ICC-LM’s spatial orientation in which the plexi follow the curved boundary between the muscle segments inside the longitudinal muscle layer (Fig. 3).

Quantitative analysis of ICC density. We have demonstrated four distinct ICC network patterns in the previous four sections. Of the four ICC subgroups, we chose to measure the density of ICC-CM to illustrate our image-based quantitation of the network components. Morphologically, ICC-CM are sandwiched in the circular muscle with an orderly and repetitive spatial arrangement (Figs. 4 and 5) that offers an appropriate target for quantitative analysis. In the following example, we use the image stack shown in Fig. 4A to illustrate our approach to ICC quantitation.

Figure 7A shows that the acquired 200-μm ICC image stack (Fig. 4A) can be divided into four sections, each with a depth of 50 μm. Projection of the 50-μm c-kit and nuclear signals into a 2-D surface allows quantitative analysis of ICC (Fig. 7B). The presence of an ICC is judged by at least two c-kit+ processes (red) encircling the cell body (green, nuclear staining) (3). Using this approach, we estimate the ICC-CM density in the sigmoid colon of the 54-year-old male subject is 2,707 ± 313 cells/mm³ (based on seven 521×521×200-μm³ image stacks).
Table 1 lists the densities of ICC-CM in four individuals, together with information about their gender, age, and location of the sampled colon segments. We observe no statistical difference between the cell densities of any two individuals, suggesting that the circular muscle of normal colon contains a steady amount of ICC-CM in 3-D space. Importantly, our result establishes a normative baseline and confirms the use of imaging approach to quantify ICC, which has been proposed as a way to evaluate pathological changes in syndrome of dysmotility (19).

In principle, the projection method can be used to quantify other classes of ICC, such as the ICC-MY, which generate the myenteric potential oscillations (17, 24, 34). Figure 8 shows the projections of ICC in and around the myenteric plexuses of the four individuals listed in Table 1. However, as indicated in Fig. 2, the ICC-MY primarily surround and attach to the ganglia, leading to variations in their spatial morphology due to the irregular ganglionic contours. Indeed, in the four projections in Fig. 8, we observe aggregates of ICC-MY, contrary to the dispersed morphology of ICC-CM in the circular muscle. Thus, care must be taken in ICC quantitation if specimens are derived from the area of myenteric plexus or its boundary, which intrinsically carries substantial variation in ICC density.

Exclusion of mast cells and identification of septal ICC using in-depth image projection. In addition to recognizing the location-dependent variation, the in-depth image projection is also capable of excluding the c-kit/H11001 mast cells in ICC quantitation to avoid overestimation of the ICC density. Figure 9, A–D provides two examples to demonstrate the resolving power of our image data to differentiate mast cells from ICC. Specifically, in the circular muscle close to the submucosa (Fig. 9, A and B) and around the septa (Fig. C and D), we found mast cells with c-kit signals encircling the cell bodies, similar to the feature of ICC. Importantly, through the in-depth image projection, mast cells can be clearly identified by their absence of processes, reducing the uncertainty of judging the cellular morphology by individual 2-D images.
Interestingly, in addition to the mast cells, we also observe the septal ICC running within the septa in Fig. 9D. These cells have been reported to conduct pacemaker activity into the circular muscle (22, 23). Taking advantage of the transparent specimen, we simultaneously used transmitted light and confocal microscopy to reveal the septa (Fig. 9E) and identify the septal ICC by their location and orientation in the projection (Fig. 9F). The examples described in this research demonstrate that the unique combination of tissue clearing, staining, and imaging makes possible in-depth visualization of different classes of ICC, which otherwise cannot be easily portrayed by the conventional microtome-based tissue analysis.

**DISCUSSION**

Image-based analysis of the gut motor-sensory apparatus (including the smooth muscle, neurons, and ICC) can help investigators examine structure patterns, offer means to explore disease mechanisms, and ultimately establish consensus on diagnosis and lead to treatment of motility disorders. In this research, we show that optical clearing enables 3-D visualization of the fluorescence-labeled networks of ICC at the myenteric plexus, longitudinal and circular muscles, and submucosal boundary up to 300 μm in depth. The deep-tissue microscopy allows 3-D illustration of the distinct network patterns/orientations of the ICC subgroups.

In 2010, the London classification of gastrointestinal neuromuscular pathology formulated a table of the relationship between clinical entities and histopathological phenotypes (19). In the table, seven clinical entities (out of 16) were listed with morphological changes in the ICC networks based on the established link between gut motility and the ICC network. However, the authors of the Gastro 2009 international working group also cautioned that using ICC imaging as a standard tool for disease analysis is still premature (18). In this research, our results are unique in that they offer two features to improve the robustness of the staining/imaging outcomes, which supports the clinical applications of ICC imaging.

First, with the use of the digital images, investigators can specify the signal-to-noise ratio of the ICC micrographs (Fig. 1, D and F). The signal peaks (i.e., the local maximum values of the signal intensity) indicate the presence of ICC processes (or cell bodies) as long as the signal-to-noise ratio follows the Rose's criterion: an object’s signal-to-noise ratio must exceed 5 to distinguish the image features with...
100% confidence (31, 42). Images with high background noise due to nonspecific binding of the c-kit antibody or bad immunostaining/imaging parameters can be instantly detected and rejected for further analysis to avoid false interpretation.

Second, in addition to the c-kit signals, cellular and tissue structures derived from the nuclear staining and transmitted light microscopy are all valuable information to generate a connected view of the ICC plexus with the associated microstructures. Particularly, nuclear staining (which can be done in 1 h) is crucial to specify the cell bodies of ICC for quantitation (Fig. 7). The additional information allowed us to reference the location and orientation of ICC network and to verify the fidelity of the signals by comparing the sources of information with each other.

By extending the tissue information in space and capturing its continuous features across the optical sections, our method provides a comprehensive and reliable way of presenting the complex 3-D ICC plexus in the specimen, compared with the conventional 2-D analysis of the tissue network. In Fig. 7 and Table 1, the image projection and cell counting of ICC-CM provide a new way for the image-based quantitation of cellular components of the ICC network. Particularly, we show that the increased depth in microscopy, at 200 μm (consisting of eighty-one 2.5-μm optical sections), enables the measurement of cell density in the scanned volume, rather than counting ICC on individual microtome cut planes, to relieve the problem of incomplete sampling. Although the 3-D method might not play an immediate role in diagnosis, this approach will provide a useful tool for both qualitative and quantitative analyses of the ICC networks and have value for studying the disease mechanism of motility dysfunction.

In summary, we integrate 3-D c-kit immunostaining, confocal imaging with optical clearing, and image rendering to reveal the network orientations of ICC subgroups (ICC-MY, ICC-LM, ICC-CM, and ICC-SM) in space and correlate them with the microstructures of the human colon wall. Future work will be aimed at extending this 3-D imaging approach to examine the motor-sensory apparatus including the neurons, glial cells, smooth muscle, and ICC in an integrated fashion. The long-term goal is to compare normal and diseased tissues and provide quantitative data for physiological and pathophysiological analyses of gut motility.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


Fig. 9. In-depth image projection helps identify mast cells (A–D) and septal ICC (C–F). A and B: mast cells in the circular muscle. These are often seen close to the submucosa (top part of the images). The specimen was derived from subject 2 in Table 1. A is 1 of the 26 images used in projection (B). Arrows indicate the mast cells; they do not have processes extending from the cell body. Transverse colon section. C and D: mast cells and ICC around and in the septa. The specimen was derived from subject 3 in Table 1. White arrows indicate the mast cells. Their morphology is similar to that of their counterparts in A and B. In addition to the mast cells, the projection also reveals the septal ICC (cyan arrows, D) lining the septa (dotted arrow, C). Transverse colon section. E and F: imaging of the septal ICC in the longitudinal colon section. The transmitted light micrograph shows the locations of the septa (red arrows) that separate the adjacent muscle segments. The in-depth image projection reveals that the septal ICC (cyan arrows) elongate and connect with ICC-MY at the myenteric plexus and run within the septa of the circular muscle layer (white arrows). E and F were taken under the same view; the red and white arrows indicate the same locations.
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