Published in final edited form as:

J Pediatr Surg. 2013 June ; 48(6): 1288–1293. doi:10.1016/j.jpedsurg.2013.03.025.

Multiphoton microscopy to identify and characterize the transition zone in a mouse model of Hirschsprung disease

Amit Aggarwal^a, Manu Jain^{a,b}, Philip K. Frykman^c, Chris Xu^d, Sushmita Mukherjee^{a,*}, and Oliver J. Muensterer^{e,*}

^aDepartment of Biochemistry, Weill Medical College of Cornell University, New York, NY-10021, USA

^bDepartment of Urology, Weill Medical College of Cornell University, New York, NY-10021, USA

^cDivision of Pediatric Surgery, Department of Surgery, Cedars-Sinai Medical Center, Los Angeles, CA-90048, USA

^dSchool of Applied & Engineering Physics, Cornell University Ithaca, NY 14853–2703, USA

^eDepartment of Surgery (Division of Pediatric Surgery), Weill Medical College of Cornell University, New York, NY-10021, USA

Abstract

Background—The distribution of ganglion cells in the transition zone of Hirschsprung Disease (HD) colons is extremely variable. Determining the resection margin based on intraoperative biopsies may be imprecise. Multiphoton microscopy (MPM) is a novel imaging technology with the ability to visualize tissues in real time. In this study, we evaluate the potential of MPM to quantify ganglion cells in a murine model of HD.

Methods—After IACUC approval, formalin-fixed colons from 7 wild type (WT) and 6 Endothelin Receptor B gene (EdnrB) homozygous knockout (KO) mice with distal colonic aganglionosis were assessed by MPM for the presence of myenteric ganglion cells. MPM images were captured starting from the anus progressing proximally at 5 mm intervals. Hematoxylin and eosin (H&E) stained biopsies of the imaged were correlated with MPM findings.

Results—WT specimens showed normal myenteric plexus ganglia throughout the examined colon. In contrast, distal colons of EdnrB KO animals were devoid of ganglia up to 10 mm from the anus. Ganglion cells were visible starting at 20–30 mm proximal to the anus. The density of ganglion cells seen by MPM and histology correlated well.

Conclusions—MPM can clearly identify the myenteric plexus ganglia in both WT and KO mouse colons. Comparison with the H&E-stained sections showed reproducible correlation. MPM-based real-time imaging of the myenteric plexus may become a useful intraoperative decision-making tool in the future.

Keywords

Hirschsprung; Ganglion cells; Multiphoton microscopy; Mouse; Autofluorescence

The distribution of ganglion cells in the transition zone of Hirschsprung disease (HD) colons is extremely variable [1]. Determining the resection margin based on a limited number of intraoperative focal biopsies can be inaccurate and lead to adverse postoperative outcome [2].

Multiphoton microscopy (MPM) is a recently developed non-linear imaging technology that utilizes femtosecond pulsed near-infrared light to visualize fresh unprocessed tissues at sub-micron resolution in real time up to a depth of several hundred microns below the specimen surface [3–5]. It captures intrinsic tissue signals based on autofluorescence and Second Harmonic Generation (SHG) from tissue components such as collagen, and when color-coded and overlaid, can provide images with information content similar to gold standard hematoxylin and eosin (H&E) histology [6–8].

This study evaluates the potential and accuracy of MPM to detect and quantify ganglion cells in a murine targeted gene knock-out model of HD [9,10] and compares the results with histopathology in the mutant and wild type control animals.

1. Materials and methods

1.1. Study design

In an IACUC-approved study (2011–0063), formalin-fixed colons from homozygous endothelin receptor B (EdnrB) knockout (KO) mice (n = 6) were assessed by MPM and histopathology. Colons from 7 wild-type (WT) mice were assessed in an identical fashion and served as controls. MPM images were captured starting from the anus going proximally up to 50 mm at 5 mm intervals. During MPM imaging, the junction between the circular and longitudinal muscular layers of the muscularis propria was identified. Myenteric ganglion cells were defined on MPM as those existing at the junction, with large, round well-defined nuclei and abundant cytoplasm. After MPM imaging, full-thickness biopsies of the imaged areas were obtained, and submitted for routine histology (paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E)). The MPM findings were then correlated with the corresponding H&E slides. Ganglion cell density was quantified by counting ganglion cells in the corresponding fields of view.

1.2. Multiphoton microscopy

The colon specimens were placed on a glass slide and fastened from and including the anus to oral end using silk sutures (Fig. 1A). They were hydrated with PBS and overlaid with a glass coverslip. A drop of PBS was placed on the coverslip to achieve water immersion of the 25×/1.05 numerical aperture objective (Fig. 1B). At each imaging point, the images were acquired as a stack of optical sections with a step size of 4 µm. Typically, images were acquired up to 250 µm below the surface. This includes the entire thickness of the mouse colon wall, from the serosal surface into the crypts. An Olympus FluoView FV1000MPE

imaging system (Olympus America, Center Valley, PA) was used for all MPM imaging. Femtosecond pulsed light at 780 nm from a tunable Ti-Sapphire laser (Mai Tai HP, Spectra-Physics, Newport Corporation, Irvine, CA) was used to excite the specimens. Two distinct intrinsic tissue emission (ITE) signals were collected using photomultiplier tubes in non-descanned configuration: (i) second harmonic generation (SHG at 360–400 nm, color coded red), a nonlinear scattering signal originating from tissue collagen, and (ii) tissue autofluorescence (420–490 nm, color coded green), originating in part from reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) in cells, and elastin in the connective tissue (Fig. 2). The field of view using this 25× objective was 0.51 mm × 0.51 mm at 0.64 μm/pixel resolution. To find the transition zone from aganglionic to ganglionic colon, a larger area of the colon wall was imaged by utilizing the automated image tiling function, which allowed multiple adjacent frames to be imaged and stitched together to produce a single, seamless, large field-of-view image. The signals in both channels were acquired as separate grayscale images and then color coded and merged to produce the final output. Metamorph, Meta Imaging Series® 7.6 (MDS Analytical Technologies, Downingtown, PA) was used for color-coding and processing of raw images. Individual frames showing the features of interest were separated from the stack of images and minor adjustment of color balance, brightness, contrast and Gaussian filtering to remove single pixel noise were made using Adobe Photo-shop CS4 (San Jose, CA).

1.3. Histopathology

After MPM imaging, full-thickness biopsies were obtained of the imaged areas, and placed in a vial containing 70% ethanol. After embedding the specimens in paraffin, 5 μm sections were cut and stained with H&E. These formalin-fixed, paraffin-embedded (FFPE) specimens were blindly assessed by the study pathologist (MJ) at Weill Cornell Medical College for the presence and density of ganglion cells.

1.4. Statistical analysis

The average number of ganglion cells per 0.51 mm × 0.51 mm field of view at particular intervals from the anus was compared between MPM and histopathology, and between KO and WT specimens using Student's t test. A p-value of <0.05 was considered statistically significant.

2. Results

Wild-type colons showed normal myenteric plexus ganglia with a consistent density throughout the examined colon. In contrast, the distal colon of KO animals was completely devoid of ganglia up to 10 mm from the anus in most animals. Typically the number of ganglia increased in number starting at 20–30 mm from the anus (Fig. 3). On the composite MPM images, the transition zone (25–30 mm from the anus in this case) showed increasing size and number of ganglion cell nests with increasing distance from the anus (Fig. 4).

While the length of aganglionosis is typically quite variable between KO animals, of the colons analyzed, the average ganglion cell density in the KO specimens remained near nil until approximately 20 mm from the anus, at which point it showed a linear increase until

approximately 35 mm from the anus where it plateaued and became similar to the WT. Not surprisingly, the ganglion cell density in the KO colons was statistically significantly lower than in WT specimens in the most distal 25 mm of colon (Fig. 5).

Comparison of relative ganglion cell density in 5 specimens of each genotype with complete datasets showed identical trends in both MPM images and histopathology slides prepared from the imaged areas (Fig. 6). The correlation coefficient between MPM and H&E was 0.92.

3. Discussion

The goal of surgical correction of Hirschsprung disease is the complete resection of the aganglionic segment of bowel. Preoperative imaging is notoriously inaccurate to detect the magnitude of aganglionosis [11], and intraoperative frozen section biopsies are cumbersome, time-consuming, and can be misleading [12,13]. Inadequate resection of the aganglionic colon can result in unfavorable outcome [2] and compromised long-term quality of life [14,15]. Additionally, intraoperative seromuscular biopsies carry significant risks, including bleeding, perforation, and peritoneal contamination [16]. Bowel perforation during biopsy has been reported in up to 4% of patients [16].

It would be immensely useful for a pediatric surgeon to have an intraoperative real-time imaging device to visualize the ganglion cells in the colon *in-vivo* to determine the precise location and extent of the transition zone between aganglionic and ganglionic colon to determine the proper location for bowel resection. Previously, Frykman et al. described an *in-vivo* spectral imaging method to accurately distinguish normal from aganglionic colon in the *piebaldlethal* mouse model of HD [17]. However, one potential drawback of this approach is the lack of a true morphologic assessment of ganglia in the tissue, since the acquired signal is a spectral wave form rather than an image.

MPM is a novel imaging modality that has been investigated for *ex-vivo* analysis of fresh tumor specimens from the human bladder [18], prostate [19] and the testis [8]. It also has been described for imaging lung [20] and gastrointestinal [21] tissues. In unpublished previous studies, we demonstrated excellent visualization of ganglion cells in the myenteric plexus of fresh (unpreserved) mouse and rat colons by MPM and optimized the imaging sequence. We also attempted imaging the submucosal plexus from the mucosal side, but results were less consistent.

In this study, we show that MPM was accurate and reproducible in identifying ganglia in the myenteric plexus when compared with the gold standard, H&E histology. Importantly, MPM was also accurate in distinguishing the presence, absence, or paucity of ganglion cells when compared to H&E histology. Interestingly, we found that the clusters of ganglia were both smaller and contained fewer cells, and were more widely spaced in the ganglionic region of the KO colons compared with the WT colons.

MPM universally showed absence or severe paucity of ganglion cells in the most distal 15 mm of colon of EdnrB knockout mice tested. There was a variable transition zone of the

adjacent proximal 10–20 mm, while the wild type mice showed a relatively even distribution of normal ganglion cell density in the examined colonic segments.

Incidentally, MPM occasionally visualized hypertrophic nerve trunks in the aganglionic bowel segments, which may be another morphologic feature for the diagnosis of HD by MPM. Since this study was not designed to assess nerve trunk size, the findings were incidental and thus, the results are not taken into account here. However, this may be included as a distinguishing feature of aganglionosis in future studies.

The limitations of our study include a small sample size. Also, formalin-fixed tissues generally yield lower signal when imaged with MPM than fresh tissue, owing to mitigated autofluorescence. However, this effect did not abolish our ability to accurately distinguish between ganglionated and aganglionated colon. Finally, movement of the bowel when translating the technique to *in-vivo* trials may add an additional challenge in maintaining adequate spatial and temporal resolution. However, *in-vivo* imaging in live, anesthetized rats has been carried out recently in the Xu laboratory using two different miniaturized MPM prototypes [22,23].

As shown in this study, MPM has the potential to provide real-time, non-invasive tissue imaging without the need for any contrast agent. The main challenge of the method for clinical applicability in the diagnosis of HD is tissue penetration. Depending on patient age, size, and thickness of the examined bowel, the myenteric plexus may be up to 400 μm from the serosal surface. Our group has achieved imaging depths of up to 1.6 mm in the mouse brain *in vivo* using exogenous contrast [4], and penetration of 500 μm at low magnification and resolution (48 $\times$ total magnification, 3.9 μm /pixel resolution) in *ex vivo* dense organs such as the bladder [19]. Therefore, we believe it is feasible to develop a miniaturized laparoscopic MPM device to image the serosal surface of the colon in neonates *in-vivo* and in real time. This prototype device is currently under development and human studies have yet to be performed at this time.

In summary, MPM is a promising imaging technique that has the potential to revolutionize HD surgery by providing the surgeon with an intraoperative, real-time diagnostic tool to detect colonic ganglion cells. Our group is working on miniaturizing an MPM device for use as a laparoscopic instrument for clinical applicability.

Acknowledgments

The authors thank Dr. Vishal Chandel for his help with the histopathological analysis.

References

1. Ieiri S, Suita S, Nakatsuji T, et al. Total colonic aganglionosis with or without small bowel involvement: a 30-year retrospective nationwide survey in Japan. *J Pediatr Surg.* 2008; 43:2226–30. [PubMed: 19040940]
2. Coe A, Collins MH, Lawal T, et al. Reoperation for Hirschsprung disease: pathology of the resected problematic distal pull-through. *Pediatr Dev Pathol.* 2012; 15:30–8. [PubMed: 22111560]
3. Kobat D, Durst ME, Nishimura N, et al. Deep tissue multiphoton microscopy using longer wavelength excitation. *Opt Express.* 2009; 17:13354–64. [PubMed: 19654740]

4. Kobat D, Horton NG, Xu C. In vivo two-photon microscopy to 1.6-mm depth in mouse cortex. *J Biomed Opt.* 2011; 16:106014. [PubMed: 22029361]
5. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. *Science.* 1990; 248:73–6. [PubMed: 2321027]
6. Zipfel WR, Williams RM, Christie R, et al. Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. *Proc Natl Acad Sci U S A.* 2003; 100:7075–80. [PubMed: 12756303]
7. Jain M, Robinson BD, Scherr DS, et al. Multiphoton Microscopy in the Evaluation of Human Bladder Biopsies. *Arch Pathol Lab Med.* 2012; 136:517–26. [PubMed: 22540300]
8. Najari BB, Ramasamy R, Sterling J, et al. Pilot study of the correlation of multiphoton tomography of ex vivo human testis with histology. *J Urol.* 2012; 188:538–43. [PubMed: 22704107]
9. Cheng Z, Dhall D, Zhao L, et al. Murine model of Hirschsprung-associated enterocolitis. I: phenotypic characterization with development of a histopathologic grading system. *J Pediatr Surg.* 2010; 45:475–82. [PubMed: 20223308]
10. Zhao L, Dhall D, Cheng Z, et al. Murine model of Hirschsprung-associated enterocolitis II: Surgical correction of aganglionosis does not eliminate enterocolitis. *J Pediatr Surg.* 2010; 45:206–11. [PubMed: 20105605]
11. Proctor ML, Traubici J, Langer JC, et al. Correlation between radiographic transition zone and level of aganglionosis in Hirschsprung's disease: Implications for surgical approach. *J Pediatr Surg.* 2003; 38:775–8. [PubMed: 12720192]
12. Romanska HM, Bishop AE, Brereton RJ, et al. Immunocytochemistry for neuronal markers shows deficiencies in conventional histology in the treatment of Hirschsprung's disease. *J Pediatr Surg.* 1993; 28:1059–62. [PubMed: 7693908]
13. Holland SK, Ramalingam P, Podolsky RH, et al. Calretinin immunostaining as an adjunct in the diagnosis of Hirschsprung disease. *Ann Diagn Pathol.* 2011; 15:323–8. [PubMed: 21641840]
14. Jarvi K, Laitakari EM, Koivusalo A, et al. Bowel function and gastrointestinal quality of life among adults operated for Hirschsprung disease during childhood: a population-based study. *Ann Surg.* 2010; 252:977–81. [PubMed: 21107107]
15. Hartman EE, Oort FJ, Aronson DC, et al. Quality of life and disease-specific functioning of patients with anorectal malformations or Hirschsprung's disease: a review. *Arch Dis Child.* 2011; 96:398–406. [PubMed: 20371581]
16. King SK, Sutcliffe JR, Hutson JM. Laparoscopic seromuscular colonic biopsies: a surgeon's experience. *J Pediatr Surg.* 2005; 40:381–4. [PubMed: 15750933]
17. Lindsley EH, Gaon M, Farkas DL. Spectral imaging for precise surgical intervention in Hirschsprung's disease. *J Biophotonics.* 2008; 1:97–103. [PubMed: 19343641]
18. Mukherjee S, Wysock JS, Ng CK, et al. Human bladder cancer diagnosis using Multiphoton microscopy. *Proc Soc Photo Opt Instrum Eng.* 2009:7161. nihpa96839.
19. Tewari AK, Shevchuk MM, Sterling J, et al. Multiphoton microscopy for structure identification in human prostate and periprostatic tissue: implications in prostate cancer surgery. *BJU Int.* 2011; 108:1421–9. [PubMed: 21443651]
20. Abraham T, Hogg J. Extracellular matrix remodeling of lung alveolar walls in three dimensional space identified using second harmonic generation and multiphoton excitation fluorescence. *J Struct Biol.* 2010; 171:189–96. [PubMed: 20412859]
21. Rogart JN, Nagata J, Loeser CS, et al. Multiphoton imaging can be used for microscopic examination of intact human gastrointestinal mucosa ex vivo. *Clin Gastroenterol Hepatol.* 2008; 6:95–101. [PubMed: 18065276]
22. Brown CM, Rivera DR, Ouzounov DG, et al. In vivo multiphoton endoscopy. *J Biomed Opt.* 2012; 17:040505. [PubMed: 22559671]
23. Huland DM, Brown CM, Howard SS, et al. In vivo imaging of unstained tissues using long gradient index lens multiphoton endoscopic systems. *Biomed Opt Expr.* 2012; 3:1077–85.

Fig. 1. The colon of an EdnrB knockout mouse with distal aganglionosis mounted for imaging (A). The anus is on the left, and the proximal colon extends towards the right. The constricted aganglionic segment clearly contrasts to the proximal dilated colon. The specimen is then placed under the Multiphoton Microscope (B) for imaging. Black marks on the specimen slide indicate 5 mm intervals.

Fig. 2.

Combined MPM image from two detection channels combining the SHG signal originating from collagen (360–400 nm, color coded red), and autofluorescence originating from NADH and FAD in cells and elastin in connective tissue (420–490 nm, color coded green). The architecture of the colon wall is clearly visible from the serosa (top) to mucosa (bottom). (Total magnification: 300×; Field of view: 0.51 mm × 0.51 mm).

Fig. 3.

Comparative MPM and H&E-stained images of mouse colon taken at corresponding intervals from the anus. Wild type (A–F) and EdnrB knockout mice (G–L). Arrows point towards clusters of ganglion cells present at the junction (a) of the two muscular layers in both MPM and H&E images. (Total magnification: MPM: 300 $\times$, H&E: 200 $\times$; Field of view: 0.51 mm $\times$ 0.51 mm).

Fig. 4.

Composite MPM image of the aganglionated to ganglionated transition zone in the colon of an EdnrB knockout mouse between 25 and 30 mm from the anus. Multiple images of adjacent overlapping frames (each 0.51 mm $\times$ 0.51 mm) were acquired and stitched together to create a "tiled" image (total magnification: 300 $\times$; field of view: 5 mm). The white arrows indicate increasingly larger and more frequent clusters of ganglion cells towards the proximal end (right). The elongated structure indicated by an arrowhead points to a blood vessel. The red signal seen on the top of the tissue constitutes the collagen fibers in the serosa.

Fig. 5. Histogram of the number of ganglion cells counted per $0.51 \text{ mm} \times 0.51 \text{ mm}$ area by MPM at increasing distance (in mm) from the anus in EdnrB knockout (KO) and wild type (WT) mice.

Fig. 6.

Comparison of average relative ganglion cell density per field of view as measured by MPM and H&E-stained histopathology slides. Only 5 specimens with complete data sets were included in this analysis. None of the differences between MPM and H&E were statistically significant (correlation coefficient 0.92).