Journal of Visualized Experiments

Diagnosis of Hirschsprung's Disease by Immunostaining Rectal Suction Biopsies for Calretinin, S100 Protein and Protein Gene Product 9.5

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58799R3
Full Title:	Diagnosis of Hirschsprung's Disease by Immunostaining Rectal Suction Biopsies for Calretinin, S100 Protein and Protein Gene Product 9.5
Keywords:	Hirschsprung's disease RBS Calretinin S100 PGP9.5 Immunostaining
Corresponding Author:	Shuiqing Chi Wuhan Union Hospital Wuhan, Hubei CHINA
Corresponding Author's Institution:	Wuhan Union Hospital
Corresponding Author E-Mail:	chishuiqing95@gmail.com
Order of Authors:	Shaotao Tang
	Shuiqing Chi
	Mijing Fang
	Kang Li
	Li Yang
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Nanjing, Jiangsu province, China

TITLE:

Diagnosis of Hirschsprung's Disease by Immunostaining Rectal Suction Biopsies for Calretinin, S100 Protein and Protein Gene Product 9.5

AUTHORS AND AFFILIATIONS:

Shuiqing Chi[1], Mijing Fang[1], Kang Li[1], Li Yang[2], Shao-tao Tang[1]

[1]Department of Pediatric Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

[2]Division of Gastroenterology, Hepatology and Nutrition, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, 45229

Corresponding Author:

Shao-tao Tang (Tshaotao83@hust.edu.cn)

Email Addresses of Co-authors:

Shuiqing Chi (chishuiqing95@hust.edu.cn)
Mijing Fang (59983637@qq.com)
Kang Li (xiaokang8290150@hust.edu.cn)
Li Yang (jennyounne@163.com)

KEYWORDS:

Hirschsprung's disease, rectal suction biopsy, calretinin, S100 protein, protein gene product 9.5, immunostaining

SUMMARY:

This protocol describes the process of immunostaining rectal suction biopsies for calretinin, S100 protein, and protein gene product 9.5. This novel adjuvant diagnostic method for Hirschsprung's disease has preferable sensitivity and specificity rates.

ABSTRACT:

Hirschsprung's disease (HD) is a congenital intestinal disease that is clinically manifested as an inability to pass meconium in infants or as long-term constipation in children. Rectal suction biopsy (RSB) to determine the absence of ganglion cells and neural hypertrophy is the most accurate test for the diagnosis of HD at present. Traditional hematoxylin-eosin staining lacks sensitivity and specificity. Acetylcholinesterase staining cannot be widely used due to its complex process. Our novel protocol of immunostaining for calretinin, S100 protein, and protein gene product 9.5 (PGP9.5), which we conducted on RSBs, exhibits high sensitivity and specificity rates of 96.49% (95% confidence interval, 0.88-0.99) and 100% (95% confidence interval, 0.97-1.00), respectively. The HD-affected segments often present as the absence of the expression of calretinin, S100 protein, and PGP9.5, which are markers of neural hypertrophy in the submucosal tissue. This protocol describes the detailed operating process of this new diagnostic method.

INTRODUCTION:

Hirschsprung's disease (HD) is a common congenital gut intestinal disorder characterized by a lack of ganglion cells in different segments of the distal intestinal tract[1]. The human enteric nervous system is formed when the invasion of the embryonic neural cells is completed. If there is a disturbance of the process and the invasion fails to complete, the distal intestine of the newborn becomes aganglionic[2]. This potentially fatal condition is called Hirschsprung's disease. Proliferation, motility, and gut growth are the three main components of successful colonization.

Traditional hematoxylin and eosin (H&E) staining of a limited submucosal biopsy cannot attain as satisfactory of a result as H&E staining of a full-thickness tissue obtained from surgery. Additionally, acetylcholinesterase (AChE) staining of rectal suction tissue is theoretically challenging due to its inadequate sensitivity, which is 91%, and complex processing of frozen sections[3,4]. Several other immunohistochemical markers of ganglion cells and nerve fibers that can be stained in formalin-fixed and paraffin-embedded specimens are gradually becoming the mainstream HD diagnostics. Calretinin is a vitamin D-dependent calcium-binding protein that is not expressed in the myenteric and submucosal plexus of HD-affected segments[5]. S100 protein is expressed in cells derived from the neural crest, such as nerve fibers and glial cells, which often present neural hypertrophy in the submucosal tissue of HD-affected segments[6]. Protein gene product 9.5 (PGP9.5) reliably stains nerve fibers and ganglion cells; PGP9.5 staining acts as a supplement to calretinin staining, especially in cases of isolated hypoganglionosis. Double staining with S100 and PGP9.5 can decrease the false-negative rate and increase the sensitivity. As a prerequisite, the current study aims to ensure adequate specificity and high sensitivity of this novel diagnostic method. Our novel protocol used all three markers for the discrimination of aganglionic intestine and hypertrophic nerve fibers. A prospective study of 318 children was performed by our lab and previously published without a detailed protocol[7]. The detailed protocol and precautions are discussed in this article. Any neonates who suffered from a severe defecation problem since birth or children with chronic constipation excluding other common diseases are potential candidates for rectal suction biopsy (RSB). Our novel protocol is suitable for staining not only RSBs but also full-thickness biopsies or surgical specimens to make a final diagnosis.

PROTOCOL:

This protocol was approved by the Research Ethics Board of the Union Hospital of Huazhong University of Science and Technology.

1. Rectal Suction Biopsy

1.1 Perform the rectal suction biopsy by a well-trained pediatric surgeon and an assistant using a Rbi2 suction rectal biopsy system after obtaining informed consent from the guardian.

1.1.1 Perform RSB on patients that have the following indications: inability to pass meconium in infants or long-term bloating with or without constipation in children; children with long-term constipation who need paraffin oil to complete defecation; intestinal obstruction or intestinal perforation with unknown reasons in children undergoing an enterostomy.

NOTE: The contraindications for RSB are as follows: children who have severe symptoms, who are in poor physical condition, or who cannot tolerant the RSB; children with acute-stage enterocolitis; children undergoing intestinal anastomosis without complete healing.

1.2 Perform a normal saline retention enema as bowel preparation 36 h before the procedure to avoid loose stools and excessive edema of the mucosa. Add magnesium sulfate solution if the child has severe constipation. Administer vitamin K (1 mg/kg) to neonates.

NOTE: Do not perform preoperative blood tests or administer antibiotics, as they are not required.

1.3 Put patient in the lithotomy position. Coat the surface of the tube with paraffin oil. Insert the blunt-ended tube 4-7 cm into the rectum with the side hole facing the posterior or lateral walls.

1.4 Apply gentle pressure on the tube to facilitate adequate adhesion of the side hole to the rectal wall. Press the trigger and withdraw the tool immediately.

1.5 Use the biopsy instrument to obtain 4 suction biopsies with a diameter of 2 mm at 3 cm and 6 cm above the dental line, anteriorly and posteriorly. Use a needle to place the specimen on gauze moistened with saline.

1.6 Observe the patient until 2 h after the procedure to rule out rectal bleeding. Avoid further rectal and anal manipulations in the first 24 h after RSB.

2. Preparation of the RSB Sections

2.1 Place the rectal suction biopsies in 4% paraformaldehyde for 6 h to fix the tissue. Use a fixative volume 5 times more than the tissue volume.

2.2 Dehydrate the tissue using the pathological tissue dehydrator for 11 h. The whole programmed process consists of the following 5 steps:

2.2.1 Place the tissue in formaldehyde and fix for 1 h.

2.2.2 Place the tissue in 75% ethanol for 4 h.

2.2.3 Place the tissue in absolute ethanol (two changes, 1 h each).

2.2.4 Place the tissue in absolute ethanol (two changes, 30 min each).

2.2.5 Place the tissue in xylene (three changes, 1 h each).

2.3 Place the tissue in paraffin wax (58-60 °C) (three changes, 1 h each). Embed the RSB tissues in paraffin blocks.

2.4 Trim the paraffin blocks using a microtome until the tissue is entirely exposed with a complete section plane.

2.5 Cut the blocks into 0.4 µm sections with a microtome.

2.6 Place the sections in 30% ethanol first and then into 45-50 °C water for a few seconds each to allow the sections to spread into a flat plate.

2.7 Transfer the paraffin sections onto slides.

2.8 Bake the slides in a 60 °C oven for 3-5 h. Avoid baking for too long, which may lead to the loss of the antigens.

2.9 Place the deparaffinized slides into xylene (2 changes, 15 min each).

2.10 Hydrate the slides in absolute ethanol, 95%, 80%, and 75% ethanol for 5 min each. Then, rinse them in reverse osmosis (RO)-purified water for another 5 min.

3. Antigen Retrieval

3.1 Make working dilutions for calretinin retrieval by mixing 4 mL of EDTA antigen retrieval buffer with 200 mL of RO-purified water. Make working dilutions for S100 and PGP9.5 retrieval by mixing 1 mL of citric acid sodium citrate buffer (100x) with 99 mL of RO-purified water.

NOTE: Calretinin can be retrieved preferentially by EDTA retrieval solution. However, citric acid sodium citrate buffer is more suitable for the retrieval of S100 and PGP9.5.

3.2 Place the slides in a coplin staining jar. Fill the jar with retrieval solution and place it into a preheated boiling water bath. After boiling for 20 min, remove the jar from the water bath and let it cool to room temperature. The cooling process takes approximately 10 min. Remove the slides and gently rinse them once with phosphate-buffered saline (PBS).

3.3 Draw a circle with a marker pen around the tissue and prepare a wet box for the following procedures.

4. Blocking

4.1 Make working dilutions by mixing 3 mL of 30% H_2O_2 with 27 mL of RO-purified water.

4.2 Add two or three drops of 3% H_2O_2 solution dropwise onto the tissue to block peroxidates. Add the H_2O_2 solution promptly to ensure that the solution will not overflow from the circle. Perform blocking of peroxidates in a 37 °C incubator for 20 min.

4.3 Rinse the slides gently with PBS (three washes, 5 min each).

4.4 Block the slides for 20 min at 37 °C with 5% bovine serum albumin (BSA, prepared by mixing 1.5 g of BSA powder with 30 mL of RO-purified water).

NOTE: Blocking with 3% H_2O_2 can prevent 95% of nonspecific staining and blocking with 5% BSA can prevent the other 5% of nonspecific staining. Combine the two methods to obtain a reliable result.

5. Antigen-antibody Reaction

5.1 Remove the 5% BSA and add 50 µL of primary antibody (calretinin, S100, or PGP9.5) to the slide. Incubate overnight at 4 °C.

5.2 Wash the slide with PBS (three times, 3 min each).

5.3 Add 50 µL of polymer enhancer (reagent A; see **Table of Materials**) to the section and incubate it for 20 min in a 37 °C incubator. Then, wash the slide with PBS (three times, 3 min each).

5.4 Add 50 µL of secondary antibody B (goat anti-rabbit/mouse IgG secondary antibody) to the section and incubate for 30 min in a 37 °C incubator. Wash the section with PBS (three times, 5 min each).

6. 3,3-Diaminobenzidine and Hematoxylin Staining

6.1 Add 850 µL of RO-purified water to a 1.5 mL tube and add the staining reagents (see **Table of Materials**) in the order A, B, and C. Add 50 µL of each reagent to obtain a total of 1 mL of 3,3-diaminobenzidine (DAB) working solution. If there are precipitates, use the solution after filtration.

6.2 Add a drop of DAB working solution to the tissue section and stain for 3-10 min. Incubate the slides in DAB at room temperature until brown staining is detected. Monitor carefully using a brightfield microscope. Keep the DAB working solution away from light. Then, wash the slide with PBS for 5 min.

6.3 Add a drop of hematoxylin to counterstain the nuclei for approximately 1 min. Then, hold the coplin jar and wash it under a trickle of water for approximately 30-40 s until the excess dye is removed. Be careful not to damage the tissue sections.

6.4 Immerse the RSB slides in a coplin jar containing PBS for 25-30 s. Then, differentiate in 1% hydrochloric acid-ethanol for 10 s. Wash the slides with PBS for 1 min.

6.5 Dehydrate the slides in the reverse order of hydration: 75%, 80%, 90%, and absolute ethanol (5 min each).

6.6 Expose the slides to xylene (2 changes, 5 min each).

6.7 Mount the coverslip using ultraclean mounting. Allow slides to dry before visualization using a microscope.

REPRESENTATIVE RESULTS:

In total, 318 patients were enrolled in our study. All of the patients underwent RSB, and the tissues were stained for calretinin, S100, and PGP9.5. The diagnosis based on our novel protocol was HD in 97 cases, non-HD in 213 cases, and suspected HD in 8 cases. Among the 132 surgical patients, 99 patients were diagnosed with HD by immunostaining of full-thickness specimens after surgery. S100 and PGP9.5 staining showed that 92% and 93% of the 99 patients, respectively, had hypertrophied nerve trunks. The other 83 of the 186 patients underwent full-thickness biopsies and showed ganglion cells. A total of 103 patients were clinically cured by conservative therapies, and HD was excluded. Of the 8 patients with suspected HD, 3 patients were categorized as short-segment HD, and the other 5 patients were categorized as non-HD.

According to our results, 97 patients were diagnosed by RSB initially. Detailed information is shown in **Table 1**. The protocol of immunohistochemical staining for calretinin, S100, and PGP9.5, which we conducted on RSBs, has high sensitivity and specificity rates of 96.49% (95% confidence interval [CI], 0.88-0.99) and 100% (95% CI, 0.97-1.00), respectively. The positive predictive value is 94.3% (95% CI, 0.87-0.99), and the negative predictive value is 99.1% (95% CI, 0.95-1.00).

Figure 1A and **1B** represent typical results after immunostaining for calretinin. Many ganglion cells, which express calretinin, can be found in the normal tissues. However, no ganglion cells were detected in any area of the tissue from the HD segment. As shown in **Figure 1C-F**, S100 and PGP9.5 immunostaining shows hypertrophied nerve trunks in the submucosa, while only granular staining of some small nerve twigs was observed in the normally innervated intestine. In our study, hypertrophic nerve trunks are nerve fibers with a diameter of ≥ 40 µm.

FIGURE AND TABLE LEGENDS:

Figure 1: Immunostaining of rectal suction biopsies for calretinin, PGP9.5, and S100. Immunostaining for calretinin in HD-affected tissue (**A**) and in ganglion cells of myenteric plexuses in normally innervated tissue (**B**). No calretinin staining was detected in the myenteric plexuses of the HD-affected segment. PGP9.5 staining in HD-affected tissue showed dense hypertrophic nerve fibers in the submucosa (**C**) in contrast to nerve fibers in the normal tissues (**D**). (**E**) Dense and prominent S100 immunostaining showed hypertrophic nerve trunks in the submucosa of HD-affected tissue. (**F**) Normal nerve fibers in the submucosa.

Table 1: Immunohistochemical staining scores for the three protein markers in rectal suction biopsies.

DISCUSSION:

Here we described a procedure using three different immunohistochemical antibodies to stain RBS sections for the diagnosis of HD. The sensitivity of our diagnostic protocol was 96.49% (95% CI, 0.88-0.99), and the specificity was 100% (95% CI, 0.97-1.00).

The most critical steps of the protocol are RSB and antigen-antibody reaction. The size of the biopsy determines the accuracy of the staining. A small biopsy will not provide enough tissue to make a precise diagnosis, while a large biopsy leads to a higher risk of bleeding. As a matter of experience, biopsies with a diameter of 2 mm are the optimal size. For the antigen-antibody reaction, proper preservation of the antibody cannot be ignored. Following the protocol step by step and gently working are needed. Reverse osmosis-purified water is recommended in all steps, yielding a clearer field for observation and making it easier for pathologists to make an accurate diagnosis.

Contrast enema, anorectal manometry, and biopsy with histology are three preoperative diagnostic methods that have been used in recent years. According to Takawira *et al.*'s research, biopsy with histology has the highest sensitivity and specificity[8]. Meier-Ruge *et al.* first reported the use of acetylcholinesterase (AChE) staining on frozen rectal suction tissue to help diagnose HD in 1972[9]. This staining method detects hypertrophic nerve trunks in the submucosa. After that report, many institutions around the world started to use this method as an adjunct for the diagnosis of HD. Due to its diagnostic limitations, researchers started to concentrate on other markers that can also stain the ganglion cells and nerve fibers in formalin-fixed and paraffin-embedded specimens. In 2004, Barshack *et al.* found that the loss of calretinin expression may help diagnose aganglionic segments in HD[10]. Kapur *et al.* and Guinard-Samuel *et al.* repeated the protocol in 2009 and concluded that immunohistochemical staining for calretinin was superior to staining for acetylcholinesterase[11,12]. In 1988, S100 immunostaining for the diagnosis of HD was first introduced by Robey *et al.*[13]. Monforte-Muñoz *et al.* repeated the method in 1998 and found that 90% of their 20 Hirschsprung cases had nerve fibers wider than 40 µm[14]. Therefore, S100 immunostaining may serve as an ancillary diagnostic method in cases without ganglion cells. Furthermore, Bachmann *et al.* noticed that immunohistochemical staining for MAP2, calretinin, GLUT1, and S100 could obtain a reliable result as accurate as frozen section techniques[15]. However, in 1992, Sams *et al.* evaluated the value of PGP9.5 in the diagnosis of HD[16]. Although S100 can detect more lamina propria nerve fibers, it has a higher false-negative rate. Therefore, we chose to use PGP9.5 and S100 together for the immunohistochemical diagnosis of HD, as previous studies recommended. Many cases of staining for neuron-specific enolase in transitional zones also showed positive ganglion cells in the myenteric and submucosal plexus, but these cells were sparse and small[12]. Similarly, calretinin-positive ganglion cells are small and have a clustered formation. A previous study indicated that some slightly thickened nerve trunks could also be detected in the transitional segments of HD, but with total colonic aganglionosis, the nerve trunks

may be within normal limits (< 40 μm)[17]. We should analyze the results case by case and combine these results with clinical manifestations to avoid misdiagnosis.

This protocol could not represent the final pathological diagnosis, so we carried out a minimum of one year of thorough clinical follow-up for each child. Additionally, this protocol requires more samples to prove its reliability.

In conclusion, the diagnosis of HD by RSB is a convenient method that provides an ideal histological diagnosis with acceptable minimal injury. The sensitivity is closely related to whether the site of sampling is correct. Immunohistochemical staining of RSB for calretinin, S100, and PGP9.5 is sensitive and specific in the detection of ganglion cells and hypertrophic nerve trunks. The combination of these three markers offers a more reliable diagnostic method for HD.

ACKNOWLEDGMENTS:

The authors thank Weibing Tang for his great help in providing the filming lab. This article is supported by the Public Welfare Research, and special funds were received from the National Health and Family Planning of China (Grant No.201402007).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Tam, P. K. Hirschsprung's disease: A bridge for science and surgery. *Journal of Pediatric Surgery*. **51** (1), 18-22 (2016).

2 Heanue, T. A., Pachnis, V. Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. *Nature Reviews Neuroscience*. **8** (6), 466-479 (2007).

3 Setiadi, J. A., Dwihantoro, A., Iskandar, K., Heriyanto, D. S., Gunadi. The utility of the hematoxylin and eosin staining in patients with suspected Hirschsprung disease. *BMC Surgery*. **17** (1), 71 (2017).

4 Agrawal, R. K. *et al.* Acetylcholinesterase histochemistry (AChE) - A helpful technique in the diagnosis and in aiding the operative procedures of Hirschsprung disease. *Diagnostic Pathology*. **10** (1), 208 (2015).

5 Kacar, A., Arikok, A. T., Azili, M. N., Ekberli Agirbas, G., Tiryaki, T. Calretinin immunohistochemistry in Hirschsprung's disease: An adjunct to formalin-based diagnosis. *The Turkish Journal of Gastroenterology*. **23** (3), 226-233 (2012).

6 Bachmann, L. *et al.* Immunohistochemical panel for the diagnosis of Hirschsprung's disease using antibodies to MAP2, calretinin, GLUT1 and S100. *Histopathology*. **66** (6), 824-835 (2015).

7 Jiang, M. *et al.* Calretinin, S100 and protein gene product 9.5 immunostaining of rectal suction biopsies in the diagnosis of Hirschsprung' disease. *American Journal of Translational Research*. **8** (7), 3159 (2016).

8 Takawira, C., D'Agostini, S., Shenouda, S., Persad, R., Sergi, C. Laboratory procedures update on Hirschsprung disease. *Journal of Pediatric Gastroenterology & Nutrition*. **60** (5), 598 (2015).
9 Meier-Ruge, W. *et al.* Acetylcholinesterase activity in suction biopsies of the rectum in the diagnosis of Hirschsprung's disease. *Journal of Pediatric Surgery*. **7** (1), 11-17 (1972).
10 Barshack, I., Fridman, E., Goldberg, I., Chowers, Y., Kopolovic, J. The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease. *Journal of Clinical Pathology*. **57** (7), 712-716 (2004).
11 Kapur, R. P. Can We Stop Looking? Immunohistochemistry and the Diagnosis of Hirschsprung Disease. *American Journal of Clinical Pathology*. **126** (1), 9-12 (2006).
12 Guinardsamuel, V. *et al.* Calretinin immunohistochemistry: a simple and efficient tool to diagnose Hirschsprung disease. *Modern Pathology*. **22** (10), 1379-1384 (2009).
13 Robey, S. S., Kuhajda, F. P., Yardley, J. H. Immunoperoxidase stains of ganglion cells and abnormal mucosal nerve proliferations in Hirschsprung's disease. *Human Pathology*. **19** (4), 432-437 (1988).
14 Monforte-Muñoz, H., Gonzalez-Gomez, I., Rowland, J. M., Landing, B. H. Increased submucosal nerve trunk caliber in aganglionosis: a "positive" and objective finding in suction biopsies and segmental resections in Hirschsprung's disease. *Archives of Pathology & Laboratory Medicine*. **122** (8), 721-725 (1998).
15 Bachmann, L. *et al.* Immunohistochemical panel for the diagnosis of Hirschsprung's disease using antibodies to MAP2, calretinin, GLUT1 and S100. *Histopathology*. **66** (6), 824-835 (2015).
16 Sams, V. R., Bobrow, L. G., Happerfield, L., Keeling, J. Evaluation of PGP9.5 in the diagnosis of Hirschsprung's disease. *Journal of Pathology*. **168** (1), 55 (1992).
17 Huang, Y., Anupama, B., Zheng, S., Xiao, X., Chen, L. The expression of enteric nerve markers and nerve innervation in total colonic aganglionosis. *International Journal of Surgical Pathology*. **19** (3), 303 (2011).

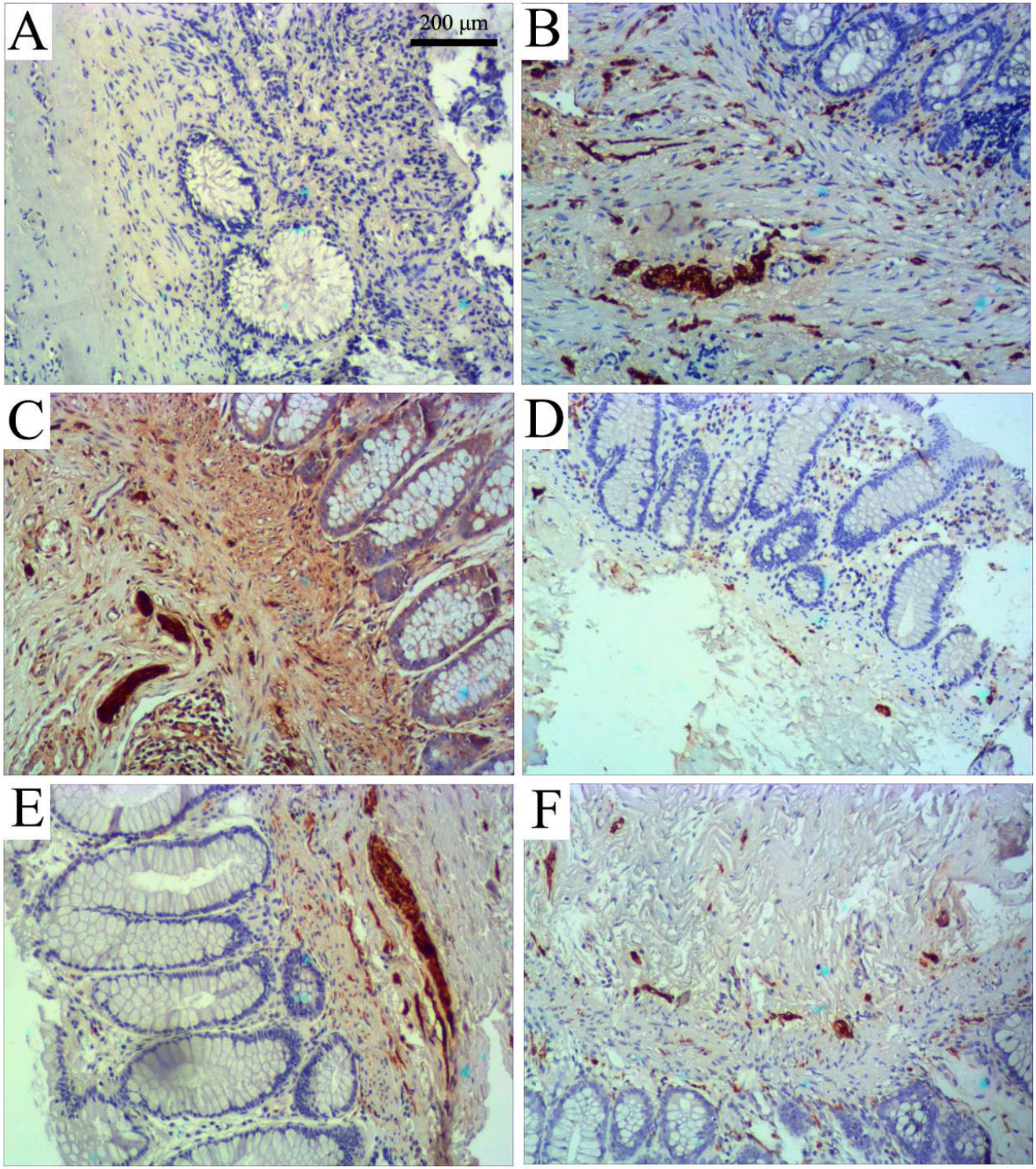


Table 1

protein markers	HD(99)	
	ganglion cells (+)	Hypertrophic nerve trunks (+)
calretinin	2	—
S100	—	91
PGP9.5	—	92

Non-HD(219)	
ganglion cells (+)	Hypertrophic nerve trunks (+)
214	—
—	3
—	4

Name of Material/ Equipment	Company
calretinin antibody	MXB Biotechnologies
S-100 antibody	MXB Biotechnologies
PGP9.5 antibody	Shanghai long island antibody Co. Ltd
enhancer reagent	MXB Biotechnologies
Goat anti-Rabbit/Mouse IgG Second	MXB Biotechnologies
DAB staining kit (containing reagent A	MXB Biotechnologies
Heat incubator	Shanghai yiheng instrument Co. Ltd
Rbi2 suction rectal biopsy system	Aus Systems Pty Ltd, South Australia, Australia
microtome	Leica
citric acid sodium citrate buffer(100X)	MXB Biotechnologies
pathological tissue dehydrator	wuhan junjie electronic Co. Ltd

	Catalog Number	Comments/Description
MAB-0716	170416405c	antibody: primary antibody
Kit-0007		antibody: primary antibody
R-0457-03		antibody: primary antibody
KIT-9902-A	170416405a	antibody: secondary antibody A
KIT-9902-B		antibody: secondary antibody B
DAB-0031		staining kit
DHP-9082		instrument
CP1200 HP1000 SS1000		instrument
leica RM2016		instrument
MVS-0101		antigen retrieval buffer
JT-12F		instrument

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Author(s):

~~Immunostaining of calretinin, S100 and protein gene product 9.5 in rectal suction biopsies~~ contribute to the diagnosis of Hirschsprung's disease
Shuiqing Chi, Mijing Fang , Kang Li , Li Yang, Shao-tao Tang

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

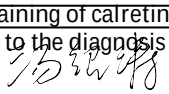
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Shaotao Tang
Department:	Department of Pediatric Surgery
Institution:	Union Hospital, Tongji Medical College Huazhong University of Science andTechnology
Title:	Immunostaining of calretinin, S100 and protein gene product 9.5 in rectal suction biopsies contribute to the diagnosis of Hirschsprung's disease
Signature:	
Date:	07/19/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Reviewers:

We wish to thank you for the time and effort you have spent reviewing our paper. We are pleased to note that you have found our research work interesting and pointed out some problems to help us improve the quality of our work.

Motivated by your comments, we have deeply reconsidered the architecture of our work and tried to fix all the problems you mentioned. In particular, this revised manuscript of our resubmitted letter has significantly been improved mainly as follows:

1. The title has been changed to Diagnosis~~e~~ of Hirschsprung's disease ~~with~~ by immunostaining rectal suction biopsies ~~of~~ for calretinin, S100 and protein gene product 9.5.
2. All the email address of authors have been changed to institutional email address except Mijing Fang.
3. The abstract has been rephrased.
4. The protocol part has been changed as required.
5. Figure 1 was uploaded with a space between the number and the units of the scale bar.
6. Acknowledgement and disclosures section were included.