Video Article

Immunostaining to Visualize Murine Enteric Nervous System Development

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Abstract

The enteric nervous system is formed by neural crest cells that proliferate, migrate and colonize the gut. Following colonization, neural crest cells must then differentiate into neurons with markers specific for their neurotransmitter phenotype. Cholinergic neurons, a major neurotransmitter phenotype in the enteric nervous system, are identified by staining for choline acetyltransferase (ChAT), the synthesizing enzyme for acetylcholine. Historical efforts to visualize cholinergic neurons have been hampered by antibodies with differing specificities to central nervous system versus peripheral nervous system ChAT. We and others have overcome this limitation by using an antibody against placental ChAT, which recognizes both central and peripheral ChAT, to successfully visualize embryonic enteric cholinergic neurons. Additionally, we have compared this antibody to genetic reporters for ChAT and shown that the antibody is more reliable during embryogenesis. This protocol describes a technique for dissecting, fixing and immunostaining of the murine embryonic gastrointestinal tract to visualize enteric nervous system neurotransmitter expression.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52716/>

Introduction

A functioning Enteric Nervous System (ENS), which controls motility, nutrient absorption, and local blood flow, is essential to life¹. The ENS is formed by neural crest cells (NCC) that proliferate, migrate and colonize the gut, where they differentiate into ganglia containing neurons and glial cells. Hirschsprung's Disease (HSCR, Online Mendelian Inheritance in Man), a multigenic congenital disorder with an incidence of 1 in 4,000 live births, can be considered the prototypic disease for studying disrupted ENS formation. In HSCR, NCC fail to migrate to and colonize variable lengths of the distal hindgut². Additionally, other common gastrointestinal (GI) developmental defects in the pediatric population, such as anorectal malformations, intestinal atresias, and motility disorders are associated with disturbances in basic ENS functions, and are likely associated with subtle, underappreciated, anatomic changes and functional changes in the ENS³⁻⁶. Therefore, techniques that allow us to understand the developmental determinants of ENS formation may shed light on the pathogenesis and potential treatment of pediatric GI tract disorders.

Following migration and colonization, NCC differentiates into neurons with markers specific for their neurotransmitter phenotype. Cholinergic neurons comprise approximately 60% of enteric neurons⁷, and can be detected by staining for choline acetyltransferase (ChAT), the synthesizing enzyme for the excitatory neurotransmitter acetylcholine. Historically, attempts to visualize cholinergic neurons were confounded by differing antigen specificity of antibodies directed against central nervous system (CNS) ChAT versus peripheral nervous system (PNS) ChAT⁸⁻¹⁰. However, antibodies directed against placental ChAT recognize both central and peripheral ChAT¹¹⁻¹³, and we have recently described techniques that allow for visualization of ENS cholinergic neurons with high sensitivity earlier in development than has been achieved with ChAT reporter lines¹⁴.

Here, we present a technique for dissecting, fixing and immunostaining of the murine embryonic GI tract to visualize ENS neurotransmitter expression in neurons. For these studies, we have utilized *ChAT-Cre* mice mated with *R26R:loxSTOP:tdTomato* animals to produce *ChAT-Cre;R26R:loxSTOP:tdTomato* mice (defined as *ChAT-Cre tdTomato* throughout the manuscript). These animals were then mated with homozygous *ChAT-GFP* reporter mice, to obtain mice expressing both fluorescent reporters that detect ChAT expression¹⁴. These two reporter animals are on a C57BL/6J background and are commercially available (Jackson Laboratories, Bar Harbor, ME).

Protocol

The University of Wisconsin Animal Care and Use Committee approved all procedures.

1. Preparation of Solutions

1. Use 1x phosphate buffered saline (PBS) as dissection buffer and rinsing solution.
2. Prepare 30% sucrose by weighing 30 g of sucrose and place into a bottle. Add 99 ml of 1x PBS and add 1 ml 10% sodium azide. Mix thoroughly until all of the sucrose is dissolved. Store at 4 °C until required.
3. Prepare Blocking solution by mixing 1x PBS, 3% bovine serum albumin (BSA) and 0.1% Triton-X-100. Mix thoroughly and store in the fridge until needed.
4. Prepare 8% paraformaldehyde (PFA) solution in 1x PBS by weighing the appropriate amount of PFA into 1x PBS and then incubate at 65 °C until it is completely dissolved. Store in 25 ml aliquots in the -20 °C freezer until needed. Dilute to 4% PFA in 1x PBS on the day of use.

2. Embryo and Gut Dissection

1. In accordance with Institutional Animal Care and Use Committee approved protocols, euthanize timed pregnant mouse and transfer the uterus into a 60 mm Petri dish containing ice cold 1x PBS.
2. Under a dissection microscope using sharp scissors, cut the uterine wall open to expose the embryos. Remove the embryos from the uterus and place into a clean 60 mm Petri dish containing ice cold 1x PBS.
3. Euthanize each embryo by decapitation in ice-cold 1x PBS. If you are using transgenic mice containing fluorescent proteins, under fluorescence illumination, identify the positive transgenic embryos.
4. Dissect the GI tract from each embryo using a dissection microscope. Using fine forceps, orient the embryo such that the left side is facing upwards and the right side is against the bottom of the Petri dish. Remove the upper body wall from the embryo to expose the internal organs. Insert fine forceps between the dorsal body wall and the internal organs. Cross the forceps against each other in a scissor-like cutting action to remove the internal organs from the embryo.
5. Further sub-dissect the GI tract away from the surrounding organs and then place each GI tract into a 1.5 ml microcentrifuge tube containing ice-cold 1x PBS.

3. Fixation of GI Tracts

1. Rinse each GI tract 3 times with ice-cold 1x PBS and then replace with 4% PFA. Fix the GI tracts in the 1.5 ml microcentrifuge tubes on a rocking platform at RT for 1.5 hr. Rinse the GI tracts 3 times for 5 min at RT and then for 1 hr on the rocking platform. At this stage, store the GI tracts at 4 °C in 30% sucrose in 1x PBS containing 0.1% sodium azide until needed.
NOTE: Alternatively, store the embryos in 30% sucrose for up to one year without any effect on the integrity of the tissues. Storage of the samples in 30% sucrose allows later processing of the samples either for immunostaining or into OCT for cryo-sectioning. Alternatively, proceed with the immunostaining protocol detailed below.

4. Immunostaining Protocol

1. If samples have been stored in 30% sucrose, rinse them 3 times for 20 min in 1x PBS on a rocking platform.
2. Place the GI tracts into blocking solution on a rocking platform for 1h at RT.
3. Remove the blocking solution and incubate the GI tracts with the appropriate amount of primary antibodies diluted in blocking solution for either 4 hr at RT or O/N at 4 °C on a rocking platform. Use 1:1,000 dilution of human anti-Hu antibody (serum obtained from patient), 1:1,000 dilution of chicken anti-green fluorescent protein (GFP) antibody and 1:100 dilution of goat anti-ChAT antibody.
NOTE: We utilize a human anti-Hu antibody that was obtained locally from a patient, however, anti-Hu antibodies are commercially available, for example, mouse anti-Hu, (use at 1:500 dilution).
4. Rinse the GI tracts in 1x PBS 3 times for 5 min and then for 1 hr at RT on a rocking platform.
5. Replace the 1x PBS with secondary antibodies diluted 1:500 in blocking solution on a rocking platform for either 4 hr at RT or O/N at 4 °C. Use donkey anti-human amine-reactive dye such as Dylight, donkey anti-chicken Cy2 and donkey anti-goat Cy5. If using the mouse anti-Hu antibody then use a 1:500 dilution of donkey anti-mouse amine-reactive dye 405.
NOTE: The intensity of the endogenous GFP and tdTomato expression and the clarity of immunostaining increase with developmental age. We typically immunostain embryonic guts individually in 0.2 ml tubes with 150 µl of staining solution in order to reduce the volume of antibody used and also to ensure efficient staining of the tissue.
6. Rinse the GI tracts in 1x PBS 3 times for 5 min and then for 1 hr at RT on a rocking platform.
7. Place a few drops of fluorescence mounting medium with DAPI onto a glass slide. Immerse the GI tract into the fluorescence mounting medium and add a cover glass directly on top of the tissue. The fluorescence mounting medium -G is a water-soluble compound that provides a semi-permanent seal.
NOTE: Use fluorescence mounting medium such as Vectashield which is a glycerol based mounting medium that prevents fading and photobleaching of antibodies. Both of these products enable tissues to be stored for long periods of time at 4 °C.
8. Capture images of each of fluorophore using a confocal microscope. Use excitation wavelengths for fluorophores and filters employed presented in **Table 3**.
9. Perform computer-aided image analysis using appropriate software depending on the type of confocal used to capture images.

Representative Results

We have previously described the generation of mice expressing both GFP and tdTomato fluorescent reporters that detect ChAT expression¹⁴. Briefly, *ChAT-Cre* mice were mated with *R26R:loxSTOP:tdTomato* animals to produce *ChAT-Cre;R26R:loxSTOP:tdTomato* mice (called *ChAT-*

Cre tdTomato). These animals were then mated with homozygous *ChAT-GFP* reporter mice. Embryos were isolated and tissues were dissected prior to being fixed and immunostained as above, with the antibodies listed in **Table 1**. The distal small intestine (SI) and proximal colon were analyzed at E11, E13.5 and E16.5

At E11, Hu-positive neurons are present within the distal SI, but NCC have not yet reached the proximal colon (**Figure 1**). At this time point, the majority of Hu-positive neurons demonstrate ChAT immunoreactivity as well as GFP positivity. Interestingly, at this time point, expression of the ChAT-Cre tdTomato construct is not detectable. By E13.5, the majority of Hu-positive neurons are both ChAT-IR and ChAT-GFP positive, and a small number of ChAT-Cre tdTomato-positive neurons are seen. The number of ChAT-Cre tdTomato-positive neurons increases by E16.5, but continues to be a small fraction of the number of ChAT-IR neurons.

Figure 1. Representative Images of Cholinergic Neurons in the Distal Small Intestine and Colon at Embryonic Day 11. At E11, in the distal SI (labeled "SI") and proximal colon (labeled "Co"), Hu-positive neurons (blue) are only present within the SI at this stage. Most Hu-positive neurons are co-immunostained with ChAT-IR (white) and ChAT-GFP (green). However, at this time point, none of these neurons are ChAT-Cre tdTomato positive (red). Scale bar = 100 μ m. [Please click here to view a larger version of this figure.](#)

Figure 2. Representative Images of Cholinergic Neurons in the Distal Small Intestine and Colon at Embryonic Days 13.5 and 16.5. Image shows cholinergic neurons in the distal small intestine at E13.5 (upper panels) and E16.5 (lower panels). At E13.5 the majority of Hu-positive neurons were ChAT-IR and ChAT-GFP positive (upper panels). A small number of these co-expressed ChAT-Cre tdTomato. By E16.5, more ChAT-Cre tdTomato neurons were found in nascent ganglia (lower panels). Scale bar = 100 μ m. [Please click here to view a larger version of this figure.](#)

Primary Antibodies			
Antigen	Immunogen	Dilution	Supplier & Details
Hu (Human neuronal protein)	Hu protein	1:1,000	Serum from human patient (Madison, WI)
GFP (Green Fluorescent Protein)	GFP protein, IgY fraction	1:1,000	Aves Lab, Inc. (Tigard, OR), Chicken polyclonal, GFP-1020
ChAT (Choline Acetyltransferase)	Human placental enzyme	1:100	Millipore (Billerica, MA), Goat polyclonal, AB144P

Secondary Antibodies			
Name	Fluorophore	Dilution	Supplier & Details
Donkey anti-Human	Dylight 405	1:500	Jackson ImmunoResearch Laboratories (West Grove, PA), 709-475-149
Donkey anti-Chicken	Cy2	1:500	Jackson ImmunoResearch Laboratories (West Grove, PA), 703-225-155
Donkey anti-Goat	Cy5	1:500	Jackson ImmunoResearch Laboratories (West Grove, PA), 705-175-147

Table 1. Details of primary and secondary antibodies used in the study. The primary and secondary antibodies used in this study, along with their supplier and the dilutions utilized.

Primary Antibodies			
Antigen	Dilution	Supplier	Secondary Antibody
Sox10	1:50	Santa Cruz Biotechnology, Dallas, TX	Donkey anti-Goat
p75	1:250	Promega, Madison WI	Donkey anti-Rabbit
PGP9.5	1:1,000	Abcam, Cambridge, MA	Donkey anti-Rabbit
BFABP	1:500	Millipore, Billerica, MA	Donkey anti-Rabbit
S-100	1:500	DAKO, Carpinteria, CA	Donkey anti-Rabbit
GFAP	1:500	DAKO, Carpinteria, CA	Donkey anti-Rabbit
nNOS	1:500-1,000	Emson, Cambridge, UK	Donkey anti-Sheep
VIP (Vasoactive Intestinal Polypeptide)	1:500	Epstein & Paulsen, Madison, WI	Donkey anti-Rabbit
Substance P	1:200-400	DiaSorin, Stillwater, MN	Donkey anti-Sheep

Secondary Antibodies			
Name	Dilution	Supplier & Details	Fluorophore
Donkey anti-Human	1:500-1,000	Jackson ImmunoResearch Laboratories, West Grove, PA	Dylight 488
Donkey anti-Human	1:1,000	Jackson ImmunoResearch Laboratories, West Grove, PA	Cy3
Donkey anti-Rabbit	1:500	Jackson ImmunoResearch Laboratories, West Grove, PA	Dylight 488
Donkey anti-Rabbit	1:500	Jackson ImmunoResearch Laboratories, West Grove, PA	Cy3
Donkey anti-Sheep	1:500	Jackson ImmunoResearch Laboratories, West Grove, PA	Cy2
Donkey anti-Sheep	1:1,500	Jackson ImmunoResearch Laboratories, West Grove, PA	Cy3
Donkey anti-Goat	1:500	Jackson ImmunoResearch Laboratories, West Grove, PA	Cy3

Table 2. Additional primary and secondary antibodies of use in studying ENS development. The primary and secondary antibodies that we have previously employed in our studies of ENS development.

Excitation			Emission	
Laser Line	Fluorophore Type	Examples	Photomultiplier Tube	Filter Options
408 nm	Blue	Alexa Fluor 405, Cascade Blue, Coumarin 30, DAPI,	1	BP 425-475 nm

		Hoechst, Pacific Blue, most quantum dots		
488 nm	Green	Alexa Fluor 488, ATTO 488, Calcein, Cy2, eGFP, FITC, Oregon Green, YO-PRO-1	2	BP 500-550 nm
561 nm	Red	Alexa Fluor 546, 555, 568, and 594, Cy3, Dil, DsRed, mCherry, Phycoerythrin (PE), Propidium Iodine (PI), RFP, TAMRA, tdTomato, TRITC	3	BP 570-620 nm
638 nm	Far Red	Alexa Fluor 633 and 647, Allophycocyanin (APC), Cy5, TO-PRO-3	4	BP 663-738 nm

Table 3. Confocal imaging excitation wavelengths, fluorophores and filters. The excitation wavelengths, detectable fluorophores, and filters employed are presented. This information can be used in choosing combinations of secondary antibodies for multicolor immunofluorescence.

Discussion

Our laboratory and others have shown that intestinal defects in HSCR are not restricted to the aganglionic colon but extended proximally, even into the ganglionated small intestine^{5,15,16}. These alterations include changes in ENS neuronal density and neurotransmitter phenotype and may account for dysmotility that has been observed in patients with HSCR. We have utilized the above techniques in our efforts to understand the determinants of ENS formation. Specifically, these techniques have been employed to visualize neuronal nitric oxide synthase (nNOS) neurons, vasoactive intestinal polypeptide neurons (VIP), as well as choline acetyltransferase (ChAT) neurons (**Table 2**)⁵. A key advantage of this technique is the ability to store samples in 30% sucrose for later processing, either for immunostaining or for embedding and cryosectioning.

NCC colonization of the gut occurs as an advancing wavefront from E9.5 to E14.5. Immunostaining with Hu, which identifies neurons, as described above, coupled with computer-aided image analysis, allows for quantitative comparisons of neuronal density. Reductions in neuronal density are found portions of the ganglionated bowel of HSCR⁵, as well as in other models of intestinal dysmotility¹³. Mice with a conditional deletion of *Hand2* demonstrate changes in the proportions of neurons expressing different neurotransmitters as well as reduced neuronal numbers. Additionally, animals with over-expression of Noggin or PTEN demonstrate increased neuronal density along their bowel^{6,17}.

The balance of neurotransmitter phenotypes among neurons of the ENS is tightly regulated, resulting in coordinated contraction of the bowel wall for movement of luminal contents, regulation of blood flow and nutrient absorption. Once NCC acquire a neuronal identity, there is a narrow window of time before committing to a neurotransmitter phenotype¹⁴. We have recently shown that immunostaining identifies a ChAT neurotransmitter phenotype at E10.5, an earlier embryonic stage than observed with genetic reporter models (e.g., ChAT-Cre or ChAT-GFP)¹⁴. Additionally, ChAT immunoreactive neurons reach adult levels (as a proportion of all ENS neurons) early in development, a result which suggests that ChAT neurons may have a role in regulating further neuronal differentiation in the ENS.

Nitric oxide synthase (NOS) is the primary relaxation neurotransmitter found in the ENS, and is proportionally increased in ENS neurons along the bowel in HSCR⁵. Additionally, alterations in vasoactive intestinal peptide (VIP), a neurotransmitter that also participates in mediating muscle relaxation and regulating blood flow along the bowel wall, are found in HSCR models⁵. These data suggest that, in order to understand how the components of the ENS regulate its function, a systematic examination of the different neurotransmitters should be undertaken. The techniques presented here can be readily adapted to allow this type of analysis by use of the appropriate antibodies.

Disclosures

The authors have nothing to disclose.

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References

- Gershon, M. D. Developmental determinants of the independence and complexity of the enteric nervous system. *Trends Neurosci.* **33**, (10), 446-456 (2010).
- Amiel, J., Sproat-Emlison, E., *et al.* Hirschsprung disease, associated syndromes and genetics: a review. *J Med Genet.* **45**, (1), 1-14 (2008).
- Erickson, C. S., Barlow, A. J., *et al.* Colonic enteric nervous system analysis during parenteral nutrition. *J Surg Res.* **184**, (1), 132-137 (2013).
- Erickson, C. S., Zaitoun, I., Haberman, K. M., Gosain, A., Druckenbrod, N. R., Epstein, M. L. Sacral neural crest-derived cells enter the aganglionic colon of *Ednrb*^{-/-} mice along extrinsic nerve fibers. *J Comp Neurol.* **20**, (3), 620-632 (2011).
- Zaitoun, I., Erickson, C. S., *et al.* Altered neuronal density and neurotransmitter expression in the ganglionated region of *Ednrb* null mice: implications for Hirschsprung's disease. *Neurogastroenterol Motil.* (2013).

6. Margolis, K. G., Stevanovic, K., *et al.* Enteric neuronal density contributes to the severity of intestinal inflammation. *Gastroenterology*. **141**, (2), 588-598 (2011).
7. Qu, Z. -D., Thacker, M., Castelucci, P., Bagyánszki, M., Epstein, M. L., Furness, J. B. Immunohistochemical analysis of neuron types in the mouse small intestine. *Cell Tissue Res*. **334**, (2), 147-161 (2008).
8. Bian, X. -C., Bornstein, J. C., Bertrand, P. P. Nicotinic transmission at functionally distinct synapses in descending reflex pathways of the rat colon. *Neurogastroenterol Motil*. **15**, (2), 161-171 (2003).
9. Johnson, C. D., Epstein, M. L. Monoclonal antibodies and polyvalent antiserum to chicken choline acetyltransferase. *J Neurochem*. **46**, (3), 968-976 (1986).
10. Tooyama, I., Kimura, H. A protein encoded by an alternative splice variant of choline acetyltransferase mRNA is localized preferentially in peripheral nerve cells and fibers. *J Chem Neuroanat*. **17**, (4), 217-226 (2000).
11. Koga, T., Bellier, J. -P., Kimura, H., Tooyama, I. Immunoreactivity for Choline Acetyltransferase of Peripheral-Type (pChAT) in the Trigeminal Ganglion Neurons of the Non-Human Primate *Macaca fascicularis*. *Acta histochemica et cytochemica*. **46**, (2), 59-64 (2013).
12. Sang, Q., Young, H. M. The identification and chemical coding of cholinergic neurons in the small and large intestine of the mouse. *Anat Rec*. **251**, (2), 185-199 (1998).
13. Lei, J., Howard, M. J. Targeted deletion of Hand2 in enteric neural precursor cells affects its functions in neurogenesis, neurotransmitter specification and gangliogenesis, causing functional aganglionosis. *Development (Cambridge, England)*. **138**, (21), 4789-4800 (2011).
14. Erickson, C. S., Lee, S. J., Barlow-Anacker, A. J., Druckenbrod, N. R., Epstein, M. L., Gosain, A. Appearance of cholinergic myenteric neurons during enteric nervous system development: comparison of different ChAT fluorescent mouse reporter lines. *Neurogastroenterol Motil*. **26**, (6), 874-884 (2014).
15. Teitelbaum, D. H., Caniano, D. A., Qualman, S. J. The pathophysiology of Hirschsprung's-associated enterocolitis: importance of histologic correlates. *J Pediatr Surg*. **24**, (12), 1271-1277 (1989).
16. Aslam, A., Spicer, R. D., Corfield, A. P. Children with Hirschsprung's disease have an abnormal colonic mucus defensive barrier independent of the bowel innervation status. *J Pediatr Surg*. **32**, (8), 1206-1210 (1997).
17. Puig, I., Champeval, D., De Santa Barbara, P., Jaubert, F., Lyonnet, S., Larue, L. Deletion of Pten in the mouse enteric nervous system induces ganglioneuromatosis and mimics intestinal pseudoobstruction. *J Clin Invest*. **119**, (12), 3586-3596 (2009).