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► **To cite this version:**

Elise Loffet, Lisa Brossard, Maxime Mahé. Pluripotent stem cell derived intestinal organoids with an enteric nervous system. Human Pluripotent Stem Cell Derived Organoid Models, pp.175-199, 2020, 10.1016/bs.mcb.2020.04.012 . hal-03327960

**HAL Id: hal-03327960**

**<https://hal.science/hal-03327960>**

Submitted on 27 Aug 2021

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**Running title:** Innervated human intestinal organoids.

## **Pluripotent Stem Cell Derived Intestinal Organoids With An Enteric Nervous System**

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Keywords: human pluripotent stem cell; intestine, enteric nervous system; vagal neural crest cell; organoid

## **Abstract**

The use of human pluripotent stem cells (hPSCs) and differentiation techniques offer new ways to generate specific tissue. It is now possible to differentiate hPSC into human intestinal organoids that include an enteric nervous system. Using step-wise differentiation processes, we generate innervated intestinal organoids that form three-dimensional structures bearing an epithelium, neurons and glial cells embedded in a supporting mesenchyme. Innervated organoids further develop to a complex structure with similar organization and cellular differentiation as the developing intestine. These tools open up new fields of application in the study of the development and pathophysiology of enteric neuropathies. Herein, we describe the generation of both human intestinal organoids and vagal neural crest cells from hPSC and their combination into an innervated organoid. We also discuss technical considerations for these experiments, and highlight advantages and limitations of the system.

## 1. Introduction

The digestive tract is a complex tubular organ responsible for digestive and absorptive function. In direct contact with the environment, it forms a selective barrier allowing the passage of nutrients and preventing the passage of pathogens. The digestive tract originates from two germinal layers: the endoderm and mesoderm, while ectoderm-derived cells that give rise to enteric neurons migrate into the intestinal tube after it has formed. Endoderm derived tissue will contribute to the epithelial lining of the gut. On the other hand, the mesoderm will form sublamina layers organized in successive tunics: the submucosal layer, the muscularis composed of longitudinal and circular smooth muscles and the serosa. This anatomical architecture ensures gastrointestinal function (Thompson et al., 2018). Although digestion and the propulsion of the food are possible because of the intestinal epithelium and the muscle, the gut motor behaviors are coordinated by an intrinsic neuronal network: the enteric nervous system (ENS).

Often called the “second brain,” the ENS is an integrated and autonomous nervous system that coordinates complex behaviors of the gut by controlling peristaltic movements and changing blood flow and secretion of water, electrolytes and hormones (Heanue & Pachnis, 2007). The developed ENS is distributed along the entire length of the digestive tract in two major plexuses (i.e. myenteric and submucosal). The myenteric neurons coordinate motor behaviours of the intestinal muscle layers i.e. gut motility. Submucosal neurons control mucosal processes *via* local reflexes that are triggered by local distension of the intestinal wall, distortion of the mucosa, and chemical contents in the lumen (Grundy & Schemann, 2007). These processes result from a direct neuronal activation but also involve enteroendocrine cells which, upon stimulation by luminal nutrients or forces, release mediators such as serotonin (Alcaino et al., 2018; Neunlist & Schemann, 2014).

Developmentally, the ENS originates from the ectoderm. The ectodermal contributions to the gut are derived from migrating vagal neural crest cells (vNCC) that primarily delaminate from the neural tube, with a minor contribution from the sacral neural crest cells (Nagy &

Goldstein, 2017). In humans, the primary gut tube is formed by 3-4 weeks of gestation, while vNCCs migrate out of the neural tube prior to week 4. By 7 weeks of gestation, the vNCCs have colonized the entire gastro-intestinal tract to differentiate in enteric neurons and enteric glial cells (Fu et al., 2004). From week 7, enteric neuronal cell bodies coalesce into ganglia to form the aforementioned plexuses (Wallace & Burns, 2005). Enteric neuronal projections make connections with other ganglia or with non-neuronal tissues to control the overall function of the gut. In addition to act as a mechanical support for enteric neurons, the enteric glia participates to the maintenance of intestinal homeostasis (Neunlist et al., 2013). The gut is anatomically mature by 11 weeks of gestation but the ENS becomes functional by the end of the first trimester and continues to develop thereafter (McCann et al., 2019).

The use of human pluripotent stem cells (hSPC) and the development of methods to differentiate them into various cell types and tissue lineages have made the *in vitro* generation of complex human tissue possible, including the generation of human intestinal organoids (HIO). Using a stepwise differentiation process that mimics embryonic intestinal development, hPSCs can be differentiated into definitive endoderm and then into hindgut spheroids that will form three dimensional intestinal structures. After 28 days in culture, HIOs possess a polarized epithelium surrounded by supporting mesenchymal cells (Spence et al., 2011). Although HIOs resemble a functional intestine, they lack an ENS. To generate innervated HIOs, hPSC-derived vagal neural crest cells (vNCC) and hindgut spheroids can be combined in a 3D human intestinal organoid with a neuronal component (HIO+ENS). These organoids are reminiscent of a normal HIO with the exception they are surrounded by neurons and glial cells (Workman et al., 2017). HIO+ENS offer new opportunities in the study of the development and pathophysiology of human enteric neuropathies.

Herein, we describe a method to produce innervated HIOs from hPSCs and we outline methods to perform immunostaining assays that can be used to characterize innervated

HIOs. We include notes on technical considerations for these experiments, and highlight key advantages and limitations of the system.

## **2. Applications of intestinal organoids with an enteric nervous system**

### **2.1. Medical need**

Several gastrointestinal diseases have a neuropathic origin where the ENS is impaired. Common enteric neuropathies include oesophageal achalasia, gastroparesis, enteric dysmotility, intestinal pseudo-obstruction, whereas more rare forms such as Hirschsprung's disease have a genetic basis and are associated with absence of enteric neurons. Often affecting children after birth, these gastrointestinal neuropathies can occur in adults subsequently to infection, immune mediated inflammation or neurodegeneration (Westfal & Goldstein, 2017). However, the pathogenetic mechanisms leading to enteric neuropathies are yet to be precisely understood. With the goal of generating a comprehensive map, comparisons between innervated organoids generated from control and diseased patients will enable the identification of dysregulated genes, pathways or cell interactions, to create insights into the pathogenesis of enteric neuropathies and potential treatments.

### **2.2. Research need**

Our ability to understand gastrointestinal physiology in particular with the ENS has been limited to animal models and cellular models that are yet to model with fidelity human physiology. The generation of a human innervated small intestine from hPSCs constitutes an approach that offers an understanding of human cellular functions and relationships with the environment and represent a significant advance in our ability to study human gastrointestinal development, physiology and diseases. The combination of organoid models, chemical and genetic perturbations and OMIC technologies including single-cell transcriptomics, represents an exciting avenue to address cell lineages and relationships, and gene networks. In addition, the transplantation of these organoids in

immunocompromised animals alleviate the possibilities to understand the development of higher order tissue with enhanced physiological output (Poling et al., 2018; Workman et al., 2017). These organoid systems bring us closer to the human physiology and hold great promises to understand basic gastrointestinal physiology in health and diseases.

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## **2. Protocol overview**

This protocol describes the generation of innervated human intestinal organoids (HIO+ENS) from hPSCs (Figure 1). Three main steps include vagal neural crest cells (vNCC) differentiation; human intestinal organoid (HIO) differentiation, followed by HIO-vNCCs co-culture in order to generate an HIO+ENS. The resulting organoid forms a 3D structure bearing a central epithelium surrounded by mesenchymal cells with enteric neurons and glial cells embedded within the mesenchyme. The overall process takes 41 days including 13 days to differentiate vNCCs, 9 days to differentiate intestinal HIOs and 28 days of HIO-vNCCs co-culture.

## **3. Step by step methods**

### **3.1. hPSC culture and maintenance prior to differentiation**

The maintenance of high quality hPSCs is crucial for differentiation and efficient production of both vNCCs and HIOs. Although many different hPSC cell lines can be used, each cell line behaves differently in culture i.e. cell growth. Culture conditions may be adjusted regarding maintenance, frequency of cell passaging and cell density at seeding prior differentiation.

#### **3.1.1. Materials and reagents**

- hPSC growing feeder-free. We routinely use the ES cell line WA01 also known as H1 (WiCell) and the iPS cell line f71.019 derived from normal human adult dermal fibroblasts (Gaignerie et al., 2018).
- Matrigel (354277, hESC-Qualified, LDEV-free, Corning)



- mTESR-1 (85850, Stem Cell Technologies)
- DMEM/F-12, HEPES (31330-038, Life Technologies)
- DPBS 1X (CS1PBS0101, Eurobio)
- StemMACS Passaging Solution XF (130-104-688, Miltenyi Biotec)
- Tissue culture-treated 6-well plate (3516, Corning)

### 3.1.2 Protocol

hPSC culture requires the pre-coating of tissue culture plates with a suitable extracellular matrix for the attachment of cells. Thus, hESC-qualified Matrigel coated 6-well plates can be prepared prior hPSC maintenance and can be stored at 4°C for a maximum of 5 days.

#### 3.1.2.1 Maintenance and passaging prior differentiation

##### *hPSC maintenance*

1. Check hPSC colonies under an inverted microscope
2. Mark differentiated cells using a non-permanent marker

*Note: We check for differentiated cells two days after passaging and everyday thereafter.*

*Pluripotent colonies present cells with a defined nucleus, they are homogeneous and have well-defined borders. Differentiated colonies present either large cells without clear colony borders or a heterogeneous zone at the center of the colony, with occasionally three-dimensional structures arising from the center.*

3. Aspirate both media and marked hPSC colonies using a pipette tip hooked to a vacuum pump
4. Add 1.5 mL of mTESR-1 per well
5. Return the plate to the incubator at 37°C, 5% CO<sub>2</sub>

### *hPSC passaging*

1. Split cells when confluence reaches 70-80%

*Note: hPSCs should be passaged at least once every 7 days.*

2. Identify and mark differentiated clusters of cells under an inverted microscope

3. Aspirate both media and marked areas using a pipette tip hooked to a vacuum pump

4. Wash with 1 mL of DPBS per well

5. Aspirate DPBS

6. Add 1 mL of StemMACS™ Passaging Solution XF per well

7. Incubate the plate at room temperature for 1 to 4 minutes depending on the cell line

*Note: cell colonies become refractive to the light under an inverted microscope when colonies start to detached from the dish.*

8. Aspirate the passaging solution when colonies' border start to lift off the dish

9. Add 1 mL of mTESR-1 per well

10. Scrape the colonies using a sterile 200µL pipette tip following a grid pattern

*Note: Using this method, colonies will be cut in even squares.*

11. Gently pipette back and forth using a 1 mL micropipette to detach cell clumps from the dish

12. Collect cell clumps into a 15 mL conical tube using a 1 mL micropipette

13. Distribute cell clumps into a hESC-qualified Matrigel pre-coated 6-well plates using a 1:10 ratio.

*Note: We typically split at a ratio of 1:10 and adjust it from 1:6 to 1:18, depending on colonies density and cell growth rate.*

14. Move the plate back and forth to evenly distribute the clumps in the well.

*Note: avoid swirling the media as colony clumps will aggregate in the center of the well, and will not grow evenly.*

15. Return the plate to the incubator at 37°C, 5% CO<sub>2</sub>

*Note: hPSCs should be kept in culture for 3 to 7 days and passaged when they reach 80% confluence.*

*Note: for passaging of hPSCs to begin differentiation of vNCCs, see section 3.2. For differentiation into HIOs, see section 3.3.*

### **3.2. Vagal Neural Crest Cells (vNCC) differentiation**

The protocol outlined here will lead to the generation of vagal neural crest cells (vNCC) in adhesion cultures in a 6-well plate format.

#### **3.2.1. Materials and reagents**

All solutions should be prepared fresh using sterile cell culture grade reagents.

- mTESR-1 (85850, Stem Cell Technologies)
- Collagenase, Type I (17018029, ThermoFisher) 500 U/mL working solution in mTESR-1
- Accutase (07920, Stem Cell Technologies)
- DMEM/F-12, HEPES (31330-038, Life Technologies)
- Neurobasal Medium (21103049, ThermoFisher)
- B-27 Supplement (50X) (17504-044, ThermoFisher)
- N-2 Supplement (100X) (17502-048, ThermoFisher)
- Penicillin-Streptomycin (15140122, ThermoFisher)
- Insulin (RP-10908, Invitrogen) 2 mg/mL stock in sterile 0.005N HCl, final concentration 5 µg/mL
- Human recombinant Fibroblast Growth Factor basic (FGF2; 130-093-840, Miltenyi Biotec), 100 µg/ml stock in sterile H<sub>2</sub>O, final concentration 20 ng/ml
- Human recombinant Epidermal Growth Factor (EGF; 130-097-749, Miltenyi Biotec), 500 µg/ml stock in sterile PBS/0.1% Bovine Serum Albumin, final concentration 50 ng/ml

- ROCK specific inhibitor Y27632 (ROCKi; 1254, Tocris Bioscience), 10 mM stock in sterile H<sub>2</sub>O, final concentration 10 μM
- GlutaMAX (35050061, ThermoFisher)
- Retinoic Acid (R2625-50MG, Sigma-Aldrich) 20 mM stock in DMSO, final concentration 2 μM
- *vNCC medium*: 50% DMEM/F-12, HEPES, 50% Neurobasal Medium supplemented with 2 mM GlutaMAX, 1X B-27 and N2 supplements, Insulin, FG2, EGF and 100 U/mL Penicillin-Streptomycin
- Fibronectin (1918-FN, R&D), 1.5 μg/mL final concentration in sterile DPBS
- 35x10 mm CytoOne Dish, Non-Treated (CC7672-3340, Starlab)

### 3.2.2 Protocol

#### 3.2.2.1 Generation of PSC-derived neurospheres (Day 0 to 6)

*Day 0 (neurosphere generation) (Fig. 2A)*

1. Remove differentiated cells from a well of hPSCs (see 3.2.1.1)
2. Wash with 2 mLs of DMEM/F12, HEPES  
*Note: Bring DMEM/F12, HEPES to room temperature for about 15 minutes and use directly.*
3. Aspirate DMEM/F12, HEPES
4. Add 1 mL of collagenase Type I prewarmed at 37°C
5. Incubate the plate for 1h at 37°C, 5% CO<sub>2</sub>
6. Confirm detachment of the colonies' border under a microscope
7. Add 5 mLs of DMEM/F12, HEPES
8. Gently flush the well using a 1 mL micropipette to detach colonies

*Note: If colonies do not fully detach, gently scrape the bottom of the plate while flushing with the 1 mL micropipette.*

9. Collect the floating cell clumps into a 15 mLs conical tube
10. Let the clumps settle in the conical tube
11. Aspirate the supernatant without disturbing the pellet
12. Wash with 5 mLs of DMEM/F12, HEPES
13. Repeat steps 10 to 12
14. Add 3 mLs of vNCC media in the 15 mLs conical tube
15. Gently resuspend the clumps using a 1 mL micropipette
16. Transfer 3 mLs of cell suspension in a sterile 35x10 mm non-treated culture dish
17. Return the dish to the incubator at 37°C, 5% CO<sub>2</sub>

*Note: The clumps will form free-floating spheres over the next day (Figure 2B).*

*Days 1 to 4 (daily media change) (Fig. 2B)*

1. Shake the dish to regroup the neurospheres in the center of the well
2. Collect the spheres into a 15 mLs conical tube using a 1 mL micropipette
3. Let the neurospheres settle in the conical tube
4. Aspirate the supernatant without disturbing the pellet
5. Add 1.5 mL of vNCC media in the 15 mLs conical tube
6. Gently resuspend the clumps using a 1 mL micropipette
7. Prepare the culture dish previously used by adding 1.5 mL of fresh vNCC media
8. Transfer 1.5 mL of sphere suspension from the 15 mLs conical tube to the dish using a 1 mL micropipette

*Note: the total volume expected per dish is 3 mLs.*

9. Return the dish to the incubator at 37°C, 5% CO<sub>2</sub>
10. Change the vNCC media daily, repeating steps 1 to 9 until day 4

*Days 4 to 6 (induction of vagal identity)*

1. Prepare 3 mLs of vNCC media per dish supplemented with 2  $\mu$ M retinoic acid
2. Shake the dish so that the neurospheres regroup in the center
3. Collect the spheres into a 15 mLs conical tube
4. Let the neurospheres settle
5. Aspirate out the supernatant without disturbing the pellet
6. Add 1.5 mL of vNCC media supplemented with 2  $\mu$ M retinoic acid in the 15 mLs conical tube
7. Prepare the culture dish previously used by adding 1.5 mL of fresh vNCC media supplemented with 2  $\mu$ M retinoic acid
8. Transfer 1.5 mL of neurosphere suspension from the 15 mLs conical tube to the dish using a 1 mL micropipette
9. Return the dish to the incubator at 37°C, 5% CO<sub>2</sub>
10. Change the vNCC media the next day, repeating steps 1 to 9

**3.2.2.2 Passaging of neurospheres on fibronectin-coated plates (Day 6 to 13)**

vNCC culture requires the pre-coating of tissue culture plates with fibronectin. Thus, fibronectin coated 6-well plates must be prepared at least an hour prior to passaging using 1.5  $\mu$ g/mL fibronectin in sterile DPBS. We recommend making 4 wells of a 6-well plate for one 35x10 mm dish of neurospheres. The pre-coated plates can be stored at 4°C for a maximum of 3 days.

*Day 6 to 10*

1. Shake plate to regroup the neurospheres in the center of the well
2. Pick and count spheres under a stereoscope

*Note: a collection of 25 to 50 neurospheres is recommended per fibronectin-coated well.*

3. Transfer 25-50 neurospheres into a 15 mLs conical tube
4. Wait for the spheres to settle in the tube
5. Aspirate the supernatant without disturbing the pellet
6. Gently resuspend 25-50 neurospheres in 1.5 mL of vNCC media
7. Transfer the neurospheres onto the fibronectin pre-coated plate using a final volume of 1.5 mL of vNCC media per well.
8. Shake the plate back and forth to evenly distribute the neurospheres in the well
9. Return the plate to the incubator at 37°C, 5% CO<sub>2</sub>

*Note: Neurospheres will adhere to the fibronectin-coated plate and form rosettes (Fig. 2C).*

10. Change the media everyday using 1.5 mL vNCC media per well until day 9

*Days 10 to 13 (passaging of neural crest cells)*

1. Aspirate vNCC media.
2. Wash with 1 mL of DMEM/F12, HEPES
3. Add 1 mL of Accutase
4. Incubate 2 min at 37°C

*Note: Confirm cell detachment under an inverted microscope. Only migrating vagal neural crest cells are picked up, the rosettes remain attached on the plate. We recommend a 2 minutes incubation with Accutase. Longer treatment will result in a detachment of the rosettes.*

5. Add 5 mLs of DMEM/F12
6. Transfer the cell suspension into a 15 mL conical tube
7. Centrifuge at 300 x g for 5 min
8. Aspirate the supernatant without disturbing the pellet
9. Resuspend the cell pellet in 1 mL of vNCC media

10. Count cells using a Malassez counting chamber or other cell counting method.
  11. Seed 180 000 single cells per well of a sterile tissue culture-treated 6-well plate (20 000 cell/cm<sup>2</sup>) in 1.5 mL of vNCC media.
  12. Shake the plate back and forth evenly to distribute the cell suspension in the well
  13. Return the plate to the incubator at 37°C, 5% CO<sub>2</sub>
- Note: Cells will adhere overnight to the bottom of the 6-well plate.*
14. Change the vNCC media everyday with 1.5 mL per well until day 13

*Beyond day 13 (passaging of neural crest cells)*

vNCCs can be passaged every 3-4 days. Thus, we repeat passaging of the neural crest cells from step 1 to 13. vNCCs maintain their phenotype up to 3 passages before combination with intestinal spheroids (*Fig. 2D*). Although we do not typically use it, the vNCCs from the first or second passages can be frozen and thawed out using standard techniques.

### **3.3. HIO differentiation and culture**

The protocol outlined here will lead to the generation of human hindgut spheroids emerging from adhesion cultures in a 24-well plate format. The spheroids will be used in combination with the vNCCs produced. We dissociate hPSC colonies into single cells rather than cell clumps to obtain a robust and reproducible spheroid generation.

#### **3.3.1. Materials and reagents**

All solutions should be prepared fresh using sterile cell culture grade reagents.

- mTESR-1 (85850, Stem Cell Technologies)
- Matrigel (354277, hESC-Qualified, LDEV-free, Corning)



- ROCK inhibitor Y27632 (ROCKi; 1254, Tocris Bioscience), 10 mM stock in sterile H<sub>2</sub>O, final concentration 10  $\mu$ M
- DMEM/F-12, HEPES (31330-038, Life Technologies)
- Accutase (07920, Stem Cell Technologies)
- RPMI Media 1640 (21875-034, ThermoFisher)
- MEM Non-Essential Amino Acids Solution (100X) (NEAA, 11140-035, ThermoFisher)
- Fetal Bovine Serum (dFBS, 26140087, Life Technologies)
- Activin A (338-AC, R&D System), 500  $\mu$ g/mL stock in 4 mM HCl, final concentration 100 ng/ml
- Human recombinant Fibroblast Growth Factor 4 (FGF4; 235-F4-01M, R&D System), 100  $\mu$ g/mL stock in sterile PBS, 0.1% BSA, final concentration 500 ng/ml
- Chiron (CHIR99021, 4423/10, Tocris Bioscience), reconstituted in DMSO at 10 mM stock in sterile DMSO, final concentration 3  $\mu$ M
- Hindgut differentiation medium: RPMI Media 1640 supplemented with 1X NEAA, 2% dFBS, 500 ng/ml FGF4 and 3  $\mu$ M Chiron
- Nunclon Delta 24-well plates (142475, ThermoFisher)

### **3.3.2. HIO culture protocol (Day 0 to 9)**

HIO differentiation requires the pre-coating of tissue culture plates with Matrigel. hESC-qualified Matrigel coated 24-well plates can be prepared prior differentiation and can be stored at 4°C for a maximum of 5 days. To seed a 24-well plate with hPSCs, we recommend using 2 wells of a 6-well plate of hPSC at 80% confluence.

*Day 0 to 2 (hPSC passaging and plating) (Fig. 3A)*

1. Remove differentiated cells from a well of hPSCs (see 3.2.1.1)

2. Wash each well to be passaged with 1 mL of DMEM/F12, HEPES

3. Add 1 mL of room temperature Accutase to each well

4. Incubate 5 minutes at 37 ° C

*Note: Cell clumps will detach from the bottom of the plate. The incubation time can be extended up to 7 total minutes if colonies remain attached.*

5. Add 2 mLs of DMEM/F12, HEPES

6. Pipette back and forth to dissociate and break cell clumps into single cells

7. Collect the cell suspension from all wells of a 6-well plate in a 15 mLs conical tube.

*Note: Proceed rapidly to the following steps to avoid excessive cell death as the enzyme Accutase is just diluted and not inactivated*

8. Centrifuge for 1 min at 300 x g

9. Aspirate the supernatant

10. Re-suspend the cell pellet with 1 mL of mTESR-1 with ROCKi

11. Count cells using a Mallasez chamber and calculate the volume needed of cell suspension to seed 24 wells of a 24 well plate

*Note: We typically seed 100 000 cells per well of a 24 well plate. Therefore, we need  $2,4 \cdot 10^6$  cells per 12 mLs of mTSEr-1 with ROCKi.*

12. Add the calculated volume of cell suspension in a 15 mLs conical tube containing 12 mLs of mTESR-1 with ROCKi

13. Distribute 500  $\mu$ L of cell suspension per well of a 24 well plate, using a 1 mL micropipette

*Note: Pipette back and forth the cell suspension in the 15 mLs conical tube every 6 wells to avoid cell sedimentation during the cell seeding*

14. Shake the plate back and forth to distribute the cells evenly in the wells

15. Return the plate to the incubator at 37°C, 5% CO<sub>2</sub> for 24 hours

16. Aspirate the media

17. Add 500  $\mu$ L of mTSEr-1 per well

18. Return the plate to the incubator at 37°C, 5% CO<sub>2</sub> for 24 hours (*Fig. 3B*)

*Day 2 to 5 (Definitive endoderm differentiation)*

1. Aspirate media on day 2
2. Add 500  $\mu$ L of RPMI Media 1640 supplemented with 1X NEAA and 100 ng/ml Activin A per well (*Fig. 3C*)
3. Aspirate media on day 3
4. Add 500  $\mu$ L of RPMI Media 1640 supplemented with 1X NEAA, 0,2% dFBS and 100 ng/ml Activin A per well
5. Aspirate media on day 4
6. Add 500  $\mu$ L of RPMI Media 1640 supplemented with 1X NEAA, 2% dFBS and 100 ng/ml Activin A per well

*Day 5 to 9 (Intestinal tissue differentiation) (Fig. 3D)*

1. Aspirate media
2. Add 500  $\mu$ L of Hindgut differentiation medium
3. Repeat steps 1 and 2 on 4 consecutive days

*Note: Spheroids may appear on day 7 or 8. In this case, use a 1 mL micropipette to discard the media instead of using a vacuum aspiration. When spheroids begin to emerge from the culture, continue to section 3.4 to combine spheroids with vNCCs.*

### **3.4. Generation of HIO+ENS**

When spheroids emerge by day 9, vNCCs can be combined prior their inclusion in a extracellular matrix gel (Matrigel).

### 3.4.1. Materials and reagents

- Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, (GFR, LDEV-free , 354230, Corning)
- DMEM/F12, HEPES (31330-038, Life Technologies)
- Accutase (07920, StemCell Technologies)
- Advanced DMEM/F-12 (12634-010, ThermoFisher)
- B-27 Supplement (50X) (17504-044, ThermoFisher)
- N-2 Supplement (100X) (17502-048, ThermoFisher)
- HEPES (15630-056, ThermoFisher)
- GlutaMAX (35050-038, ThermoFisher)
- Penicillin-Streptomycin (15140122, ThermoFisher)
- Human recombinant Epidermal Growth Factor (EGF; 130-097-749, Miltenyi Biotec), 500 ug/ml stock in sterile PBS/0.1% Bovine Serum Albumin, final concentration 50 ng/ml
- *Minigut media*: Advanced DMEM/F-12 supplemented with 1X B27 and 1X N2 supplements, 10 mM HEPES, 2 mM GlutaMAX, 100 U/mL Penicillin-Streptomycin and 50 ng/ml EGF
- Nunclon Delta 24-well plates (142475, ThermoFisher)

### 3.4.2. Protocol

#### *Recovery of intestinal spheroids*

1. Recover floating spheroids using a 1 mL micropipette under a stereo-microscope

*Note: While collecting spheroids, we recommend that a rough estimate of the number of spheroids is obtained, in order to determine the number of vNCCs that will be required.*

2. Collect the spheroids in a 15 mLs conical tube. Multiple wells from a 24 well plate can be combined into one tube.
3. Wait for the spheroids to settle in the tube
4. Aspirate out media
5. Add 1 mL of DMEM/F12
6. Place the 15 mLs conical tube in an incubator and proceed to the recovery of vNCCs

#### *Recovery of vNCCs*

*A well of a 6-well plate of vNCCs usually contains around  $0,4-0,5 \cdot 10^6$  cells. We recommend using a ratio of 1000 vNCCs per spheroid.*

1. Aspirate media
2. Wash with 1 mL DMEM/F12
3. Add 1 mL Accutase
4. Incubate 2-3 min at 37°C.
5. Add 5 mLs of DMEM/F12
6. Transfer the cell suspension in a 15 mLs conical tube.
7. Centrifuge at 300 x g for 5 min.
8. Aspirate supernatant without disturbing the pellet
9. Resuspend the cell pellet in 1 mL DMEM/F12
10. Count cells using a Malassez counting chamber or other cell counting method.
11. Proceed to the co-culture of intestinal spheroids and vNCCs

#### *Co-culture of intestinal spheroids and vNCCs (Fig. 4A-D)*

1. Add the required volume of vNCC suspension to the 15 mLs conical tube containing the spheroids

*Note: We recommend using a ratio of 1000 vNCCs per spheroid.*

2. Centrifuge 2 min at 300 x g
3. Aspirate supernatant
4. Gently resuspend the spheroids and vNCCs in ice-cold Matrigel, pipetting up and down gently to evenly mix vNCCs with spheroids.

*Note: Approximately 20 to 40 spheroids per 20  $\mu$ L of Matrigel can be embedded. Matrigel should be kept on ice at all time to avoid polymerization.*

5. Distribute 20  $\mu$ L droplets of ice-cold Matrigel (without vNCCs+spheroids) in the center of a 24-well plate.

*Note: We realize a Matrigel bed first to avoid vNCC accumulation at the bottom of the plate.*

6. Overlay the existing Matrigel drop with 20  $\mu$ L of hindgut spheroids+vNCCs Matrigel suspension
7. Allow Matrigel to start polymerization for 2-3 minutes at room temperature
8. Turn the cell culture plate upside-down
9. Return the plate to the incubator at 37°C, 5% CO<sub>2</sub> for 20 to 30 minutes
10. Overlay the Matrigel drop with 500  $\mu$ L of Minigut media
11. Replace media twice a week for 14 days

#### *Passaging of innervated intestinal organoids*

Organoids should be passaged after 2 weeks of culture and placed in fresh Matrigel.

1. Collect the Matrigel drop containing HIO+ENS with a 1 mL micropipette under a stereomicroscope using sterile culture condition

*Note: 1 mL pipette tips can be cut to facilitate HIO+ENS removal from the Matrigel.*

2. Transfer HIO+ENS in a sterile petri dish filled with DMEM/F12, HEPES
3. Under a stereomicroscope, separate HIO+ENS from remnant Matrigel using fine forceps
4. If multiple HIO+ENS structures appear to be fused together, separate them into individual structures using fine forceps

*Note: Under the stereomicroscope, single HIO+ENS can be selected and separated by identifying a central epithelial core.*

5. Collect HIO+ENS in a 15 mLs conical tube

6. Allow HIO+ENS to settle to the bottom of the tube

*Note: If HIO+ENS do not settle, briefly spin the tube at 300 x g.*

7. Aspirate supernatant

8. Resuspend HIO+ENS in ice-cold Matrigel

*Note: Approximately 5-10 HIO+ENS per 35  $\mu$ L of Matrigel can be embedded.*

9. Distribute 35  $\mu$ L of Matrigel containing HIO+ENS in the center of the well of a 24-well plate.

10. Allow Matrigel to start polymerization for 2-3 minutes at room temperature

11. Turn the cell culture plate upside-down

12. Return the plate to the incubator at 37°C, 5% CO<sub>2</sub> for 20 to 30 minutes

13. Overlay the Matrigel drop with 500  $\mu$ L of Minigut media

14. Replace media twice a week for 14 days

### **3.5. Differentiation benchmarking**

Differentiation of cell types can be monitored using a combination of both gene or/and protein expression. For example, definitive endoderm can be assessed by controlling the proportion of CXCR4-expressing cell by flow cytometry or FOXA2 and SOX17 positive cells using immunofluorescence. Similarly, the expression of CDX2 can be verified in hindgut spheroids. Finally, the expression of CD49d, HNK-1 and P75<sup>NTR</sup> expression can be tested in vNCCs. Common markers used for benchmarking the differentiation steps for both HIOs and vNCCs are indicated in Table 1. The provided list is not exhaustive and additional markers can also be used to assess the differentiation.

### 3.6. Analysis – Fluorescent immunostaining

The following protocol describes immunostaining on paraffin-embedded sections of intestinal organoids. This technique may be used for imaging of specific targets or for quality control during and after differentiation.

#### 3.6.1. Materials and reagents

- DPBS 1X (CS1PBS0101, Eurobio)
- Paraformaldehyde, 2% diluted in DPBS
- Glycine (50046, Sigma-Aldrich)
- Histogel (HG-4000-012, ThermoFisher)
- Xylene
- Butanol
- Ethanol 100%, 95%, 90%, 75% and 70%
- Dako target retrieval solution 10X (S1699, Agilent)
- Bovine Serum Albumin (BSA, A7030, Sigma-Aldrich)
- Triton X-100 (X100, Sigma-Aldrich)
- Tween-20 (P9416, Sigma-Aldrich)
- Horse serum (16050122, ThermoFischer)
- Rabbit anti-CDX2 antibody (ab227201, Abcam)
- Mouse Anti-E-cadherin antibody (MAB7481, R&D Systems)
- Anti-rabbit Alexa Fluor 568 (A10042, ThermoFischer)
- Anti-rabbit Alexa Fluor 488 (A-21206, ThermoFischer)
- DAPI (D1306, ThermoFischer)
- *Washing solution*: DPBS supplemented with 0.5% Triton X-100, 0.05% Tween 20, 0.1% BSA



- *Blocking solution*: DPBS supplemented with 0.5% Triton X-100, 0.05% Tween 20, 0.1% BSA and 10% horse serum
- Prolong glass antifade mounting solution (P36982, ThermoFisher)
- Tissue processor
- Paraffin embedding station
- Pressure cooker

### 3.6.2. Protocol

#### *Organoid processing and paraffin section*

1. Aspirate media and add 500  $\mu$ L of a 2% PFA solution to each well
2. Incubate for 2 hours at 4°C
3. Aspirate PFA solution
4. Wash with 500  $\mu$ L of DPBS/0,75% Glycine, under agitation for 10 minutes
5. Repeat the procedure 3 times
6. Retrieve HIO+ENS out of well
7. Embed 3-4 HIO+ENS in 30  $\mu$ L of Histogel
8. Transfer HIO+ENS into an appropriately labeled tissue processing embedding cassette.
9. Place samples in a tissue processor and proceed overnight to a standard tissue

processing protocol:

- 70% Ethanol x 2 for 30 min each
- 75% Ethanol for 1 h
- 90% Ethanol for 1 h
- 95% Ethanol for 1 h
- 100% Ethanol x 2 for 1 h each
- 100% Ethanol for 20 min

-100% Butanol for 20 min

-Xylene x 3 for 1 h each

-Paraffin x 3 for 1 h each

10. Remove samples from tissue processor and embed them into appropriately sized disposable base molds
11. Discard the lid of the cassette and affix the bottom portion on top of the mold creating a tissue block
12. Remove the disposable base mold from the paraffin tissue bloc
13. Section into the block slightly, exposing the desired tissue face
14. Rehydrate the tissue soaking in a bath of PBS for 1 h at room temperature (RT), before chilling on ice
15. Section the tissue into ribbons 3  $\mu\text{m}$  thick and float sections until wrinkles disappear in a tissue flotation bath
15. Mount sections on to microscope slides
16. Bake the microscope slides at 60  $^{\circ}\text{C}$  for 1 h
17. Store microscope slides at room temperature until further use

### *Staining*

1. Warm microscope slides 30 min at 65 $^{\circ}\text{C}$ .
2. Rehydrate microscope slides by immersing them in the following series of baths:
  - Xylenes x2 for 7 min each
  - 100% EtOH for 5 min each
  - 95% EtOH for 4 min
  - 70% EtOH for 3 min
  - Deionized water x2 for 2min
3. Perform heat induced epitope retrieval in Dako Target retrieval solution for 1 min 30s at 110 $^{\circ}\text{C}$

4. Permeabilize organoid sections in 0.5% Triton X-100/PBS at room temperature for 30 min
5. Wash in wash solution for 10 min with gentle agitation
6. Repeat the procedure 2 times
7. Outline tissue sections with a hydrophobic pen to contain reagents
8. Wash slides in wash solution for 10 min each with gentle agitation
9. Repeat the procedure 3 times
10. Incubate slides with blocking solution for 1 h in a square bioassay dish lined with a damp paper towel to maintain humidity in the chamber
11. Aspirate blocking solution

Note: Do not wash slides.

12. Incubate slides with rabbit anti-CDX2 at [1:500] and mouse anti-CDH1 at [1:500] in blocking solution at 4 °C overnight in humidity chamber
13. Wash slides in wash solution for 10 min each with gentle agitation
14. Repeat the procedure 3 times
15. Incubate slides with donkey anti-rabbit Alexa Fluor 568 (binds to anti-CDX2 antibody) and donkey anti-mouse Alexa Fluor 488 (binds to anti-CDH1 antibody) at [1:500] in blocking solution at 4 °C overnight in a humidity chamber.
16. Wash slides in washing solution for 10 min with gentle agitation while protected from light
17. Repeat the procedure 3 times
18. Incubate slides with 1 µg/mL of DAPI in PBS for 10 min while protected from light
19. Wash microscope slides in PBS for 3 x 10 min each, while protected from light
20. Mount microscope slides with coverslips using Prolong mounting solution, or another aqueous mounting medium

*Note: Wait at least 24 hours to image slides.*

21. Proceed to imaging and cell counting under a fluorescent microscope (Fig. 5).

## **4 Troubleshooting & Optimization**

Problems may occur during the differentiation and culture processes. We listed below possible solutions to overcome these issues.

<b>Problem</b>	<b>Solution</b>
The definitive endoderm monolayer is not confluent	You can adjust hPSC cell starting density and/or control for Activin A activity
The yield of hindgut spheroid is low	You need to adjust PSC cell starting density
Neurospheres do not adhere on the plates	The fibronectin pre-coating is not working. Plates must be coated at least an hour prior the experiment
vNCC do not differentiate into enteric neurons and glial cells within the HIO	The number of vNCC is either too low or high. Although, a range of vNCC densities can be used, we recommend using 1000 vNCCs per hindgut spheroid
HIO+ENS are growing flat in the culture plate	The organoids start to adhere and grow on the plastic. When HIO+ENS are embedded in Matrigel, we recommend inverting the plate during the polymerization process
HIO+ENS do not have an epithelium after 28 days in culture	It is possible that mesenchymal cells have outgrown the epithelium. Using fine forceps, we recommend to remove excess of mesenchyme during the passaging procedure.

hPSC cell lines may present some differentiated cells if unchecked, thereby introducing unwanted cell types during the differentiation process. For these reasons, we recommend to thoroughly check and remove differentiated cells/colonies prior to starting the protocol. If the

unwanted spontaneous differentiation increases in hPSC cultures across passages, we suggest to discard the current cells and thaw out a lower passage cryovial of undifferentiated hPSCs.

Optimal conditions for intestinal differentiation may vary for different pluripotent stem cell lines. The starting cell density is a critical aspect that needs to be tested for every hPSC line. This range is variable but a reasonable starting point is 100,000 cells per well of a 24-well plate. We recommend testing several densities ranging from 80,000 to 140,000 cells for each differentiation. The success of differentiation can be approximated by the hindgut spheroid yield. For example, one can reasonably assume that if very few spheroids are produced, the differentiation process was sub-optimal, and the endoderm and hindgut differentiation will need to be optimized. As hPSC lines may vary across differentiation, we recommend using well-established ES lines as a gold-standard for these experiments. Thus, H1 (WA01, Wicell) or H9 (WA09, Wicell) human embryonic stem cells consistently give robust differentiation into intestinal organoids and can be used as positive controls for other iPSC lines.

Sources of reagent and lot testing are also critical for reproducibility across experiments. Recombinant protein or small molecule activity may differ. Thus, lot testing is highly recommended especially for Activin A, FGFs and CHIR99021. The testing can be performed using the aforementioned control markers during the differentiation process.

## **5. Precursor and alternative techniques**

Previous groups have achieved the directed differentiation of human intestinal organoids (Spence et al., 2011). Although, we primarily focused this method on the generation of small intestinal tissue from hPSCs, organoids can be differentiated to form specific regionalized areas of the gut. The directed differentiation using combined anterior-posterior signaling allows us to generate both foregut and hindgut tissues. Hence, the temporal activation of WNT/FGF/BMP/Retinoic acid/EGF signaling during the differentiation process can drive the

tissue's fate toward oesophagus (Trisno et al., 2018), stomach (McCracken et al., 2014, 2017), jejunum- to ileum-like tissues (Tsai et al., 2017) or colon (Múnera et al., 2017). Based on the generation of human intestinal organoid, we can engineer in an ENS using enteric vNCCs. Using similar protocols, previous groups have demonstrated the directed differentiation of hPSCs into neural crest cells (NCC) (Bajpai et al., 2010; Curchoe et al., 2010) including enteric vagal neural crest cells (vNCC) (Barber et al., 2019; Fattahi et al., 2016; Lau et al., 2019). Interestingly, hPSC-derived vNCCs can be combined within different gut regions including the colon (Lau et al., 2019).

As we translate embryological development in a dish, the maturation stage of the organoids reflects the corresponding embryonic stage and is reminiscent of a fetal intestine (Finkbeiner et al., 2015). This lack of maturation may represent an issue as far as the comparability of these engineered organoids to postnatal human tissue. *in vivo* engraftment of HIO in immunocompromised mice can be used to further tissue expansion, maturation and function (Watson et al., 2014). Transplanted HIO+ENS similarly develop into a mature intestinal tissue with crypts, villi, laminated subepithelial, and mesenchymal layers. Strikingly, these HIO+ENS exhibit similar features to the human ENS including neuronal bundles reassembling a myenteric-like plexus and a peristaltic like activity (Workman et al., 2017).

## **6. Summary**

The technique described in this protocol leads to the production of innervated human intestinal organoids. The key steps include the parallel differentiation of intestinal spheroids and vNCC from hPSC, and their combination into a self-assembled innervated intestinal organoid.

Using this organoid system coupled with perturbations of developmental pathways will allow us to directly address developmental processes and disease-related mechanisms. This includes both acute and chronic enteric neuropathies. In addition, patient specific or gene edited iPSC lines, can also be used to model tissue specific diseases. For example, a

mutation in the Paired-like homeobox 2b (PHOX2B) gene has been associated with Hirschsprung's disease and was successfully reproduced with the HIO+ENS (Workman et al., 2017).

Although HIO+ENS are a step forward in generating intestinal tissue with greater functionality, important facets including an immune system or bacteria are absent. Strategies wherein the immune system is added to HIO+ENS will further improve the system. The transition of a sterile gut to colonization by a microbiome, using dedicated techniques, is also an important consideration (Hill et al., 2017). Finally, surgical strategies with transplanted HIOs offer opportunities to shed light on the role of the luminal content in intestinal maturation and gut function (Mahe et al., 2017).



## **Acknowledgments**

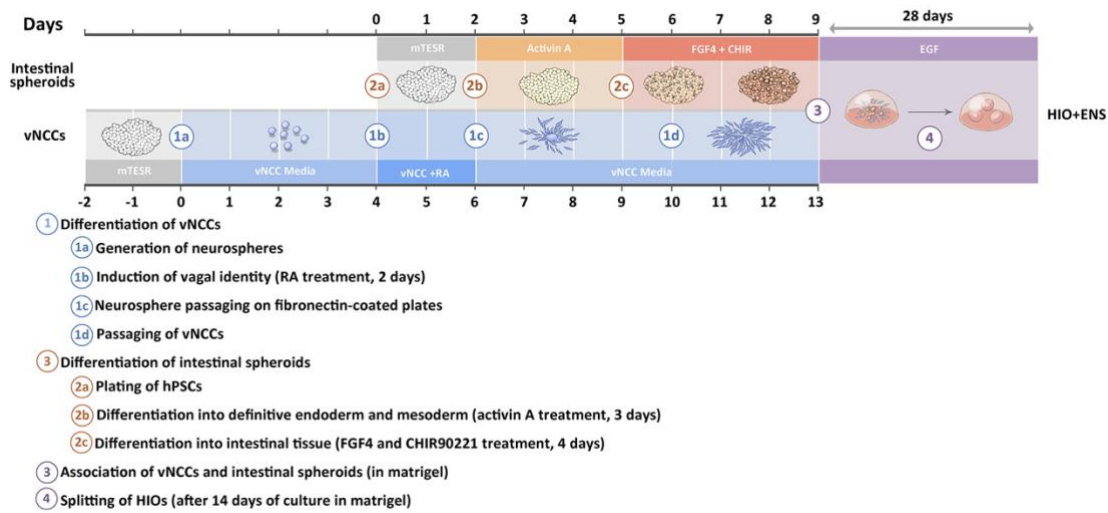
This work was supported in part by ANR-17-CE14-0021 (SyNEDI to M.M.M.) and a “New Team” grant (BOGUS to M.M.M.) from the Bioregate Regenerative Medicine Cluster, University of Nantes and Région Pays de la Loire.

**Table legends:**

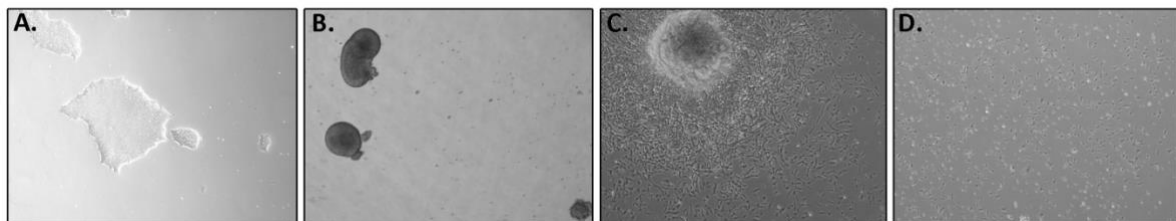
Section	Cell type	Markers	Analysis
3.2.2.2	vNCCs	HNK-1 (CD57)	qPCR, IF
		Neurotrophin Receptor p75 (p75NTR)	qPCR, IF
		SRY-Box Transcription Factor 10 (SOX10)	qPCR, IF
		Integrin Alpha 4 (CD49d)	FC
3.3.2	definitive endoderm	SRY-Box Transcription Factor 17 (SOX17)	qPCR, IF
		Forkhead Box A2 (FOXA2)	qPCR, IF
3.3.2	mid/hindgut spheroids	Caudal Type Homeobox 2 (CDX2)	qPCR, IF
3.3/3.4	epithelial cells	Epithelial Cell Adhesion Molecule (EPCAM)	IF
		E-Cadherin (CDH1)	IF
3.3/3.4	fibroblasts	Vimentin (VIM)	IF
3.3/3.4	myofibroblasts	Alpha Smooth Muscle Actin (ACTA2)	IF
3.4	glial cells	Glial Fibrillary Acidic Protein (GFAP)	IF
		S100 Calcium Binding Protein B (S100B)	IF
3.4	neurons	Tubulin Beta 3 Class III (TUBB3)	IF
		Ubiquitin C-Terminal Hydrolase L1 (UCHL1)	IF

**Table 1: Markers used for benchmarking the differentiation steps.** A combination of markers can be used to confirm the efficiency and quality of differentiation using semi-quantitative PCR (qPCR), immunofluorescence staining (IF) or flow cytometry (FC).

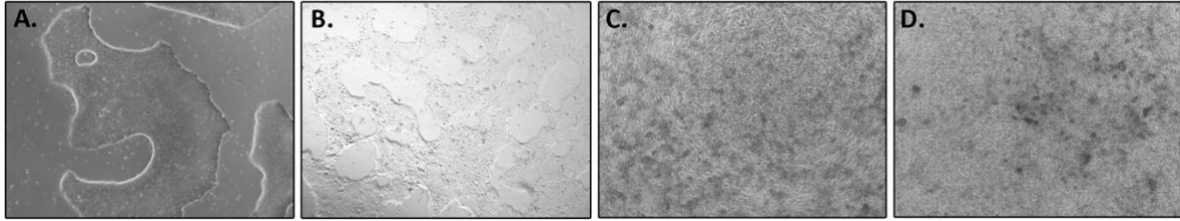
## Figure legends:



**Figure 1: Innervated human intestinal organoids differentiation protocol overview.** The experiment starts with the vagal neural crest cells (vNCC) differentiation, followed by the human intestinal organoid generation (HIO). Association of both vNCCs and HIOs is performed at day 13. The combined cells result in the self-assembled intestinal organoid containing enteric glia and neurons (HIO+ENS).

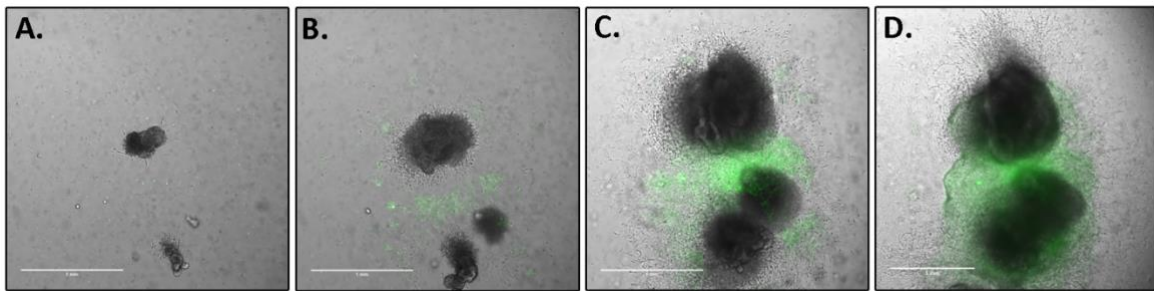


**Figure 2: Representative images of vagal neural crest cell differentiation.** A. hPSCs prior vNCC differentiation. B. Floating neurospheres (day 3). C. vNCCs migrating out of a neural rosette (day 9). D. vNCCs in culture after one passage (day 11).



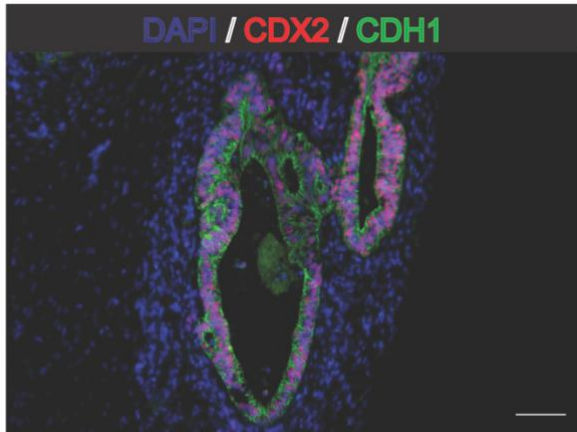
**Figure 3: Representative images of hindgut spheroid differentiation.**

**A.** hPSCs prior differentiation. **B.** Definitive endoderm differentiation (day 7). **C.** Cells forming tridimensional ridges during hindgut specification (day 10). **D.** End of intestinal spheroids differentiation (day 12). Spheroids are detaching from the adherent cell layer.



**Figure 4: Growth of innervated intestinal organoid in Matrigel over 13 days.**

GFP-expressing vagal neural crest cell differentiation (vNCC) are showed in green. **A.** Innervated intestinal organoid (HIO+ENS) a day after the combination of hindgut spheroids and vNCCs. **B.** HIO+ENS after 5 days in culture. **C.** HIO+ENS after 9 days in culture. **D.** HIO+ENS after 13 days in culture prior splitting. (Scale bars: 1 mm)



**Figure 5: Immunostaining of an intestinal organoid grown for 28 days in Matrigel.**

The intestinal organoid forms a central epithelium stained for E-cadherin (CDH1, green), CDX2 (red) and nuclei (DAPI, blue). A supporting mesenchyme surrounds the epithelium. (Scale bar: 50 $\mu$ m)

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