HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2018 September 30.

Published in final edited form as:

Methods Mol Biol. 2019 ; 1576: 229–247. doi:10.1007/7651_2017_1.

Human Intestinal Enteroids: New Models to Study Gastrointestinal Virus Infections

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Abstract

Human rotavirus (HRV) and human norovirus (HuNoV) infections are recognized as the most common causes of epidemic and sporadic cases of gastroenteritis worldwide. The study of these two human gastrointestinal viruses is important for understanding basic virus-host interactions and mechanisms of pathogenesis and to establish models to evaluate vaccines and treatments. Despite the introduction of live-attenuated vaccines to prevent life-threatening HRV-induced disease, the burden of HRV illness remains significant in low-income and less-industrialized countries, and small animal models or ex vivo models to study HRV infections efficiently are lacking. Similarly, HuNoVs remained non-cultivatable until recently. With the advent of non-transformed human intestinal enteroid (HIE) cultures, we are now able to culture and study both clinically relevant HRV and HuNoV in a biologically relevant human system. Methods described here will allow investigators to use these new culture techniques to grow HRV and HuNoV and analyze new aspects of virus replication and pathogenesis.

Keywords

Gastrointestinal viral infections; HIEs; Human intestinal enteroids; Human norovirus; Human rotavirus

1 Introduction

Diarrheal diseases are an important global health problem, causing an estimated 4 % of all deaths worldwide, including 1.3 million deaths per year for all ages with an estimated 499,000 childhood deaths in developing countries [1, 2]. Diarrheal disease can be caused by many pathogens including bacteria, parasites, and viruses, with the latter not being controllable through improvements in water, sanitation, and hygiene. Thus, viral gastroenteritis remains an important public health threat.

Human rotavirus (HRV) and human norovirus (HuNoV) are the most frequent causes of acute gastroenteritis worldwide [3–6]. Historically, HRV was the most common cause of severe diarrheal disease and the leading cause of diarrhea-related deaths in children [7]. The use of live-attenuated HRV vaccines has made a tremendous impact in reducing HRV disease in the developed world [8]. However, the efficacy of the HRV vaccines remains low in less-industrialized world [9]. Thus, many children continue to suffer from HRV-related disease today, and long-term consequences of diarrheal disease are increasingly being recognized [10]. With HRV disease decreasing markedly in developed countries, HuNoV

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infections have emerged as the most common cause of epidemic and sporadic cases of acute gastroenteritis worldwide in children [3, 11]. In addition, HuNoVs affect all age groups and are the leading cause of food-borne gastroenteritis. The major barrier to research and development of effective interventions for HuNoVs has been the lack of a robust and reproducible in vitro cultivation system. Such a system is critical to achieve a full mechanistic understanding of HuNoV replication, stability, evolution, pathogenesis, and vaccine development [12].

In 2007, Dr. Hans Clevers' group identified Lgr5 as a marker for stem cells in the intestinal epithelium in mice and showed multicellular intestinal epithelial cultures could be established ex vivo from isolated Lgr5+ cells [13]. With this knowledge, they subsequently developed human intestinal enteroids (HIEs), where three-dimensional (3D) in vitro cultures are derived from proliferating stem cells in crypts isolated from human gastrointestinal (GI) tract biopsies or surgical specimens [14, 15] (Fig. 1a). HIE cultures possess the multicellular complexity and organization of the intestinal epithelium and are physiologically active [16]. They contain all epithelial cell types of the normal GI tract and retain segment-specific properties [17]. These remarkable cultures are being developed for many basic, clinical, and translational applications, although infectious disease research was not highlighted initially [14]. With the advent of the HIEs, we evaluated and have shown the relevance of these novel cultures to model both HRV and HuNoV infections [12, 18, 19]. Importantly, we have shown for the first time that HIEs support replication of multiple strains of HuNoV as well as HRVs better than animal rotavirus strains, thus recapitulating the known biology of these two important human pathogens. Culturing each of these viruses requires both HIEs and the addition of intestinal factors to obtain optimal replication.

In this chapter, we describe our methods for the maintenance, expansion, and differentiation of 3D and monolayer HIEs as well as their utilization in HRVand HuNoV infections (Fig. 1).We do not describe the establishment of these cultures from patient tissues because this has been reported previously in several methods papers [12–15, 18–20]. We detail an HRV infection method using differentiated 3D HIEs in the presence of added pancreatin. Because of HRV's affinity toward differentiated cell types, proper and controlled differentiation of the 3D HIEs is crucial for successful infection. With HIE differentiation, HRV is able to infect without mechanical disruption, and the infected HIEs can be maintained in suspension until analysis [18]. The method for HuNoV infection uses monolayer or transwell cultures of HIEs and the addition of bile that affects the cells [12]. Different culture conditions also are needed to achieve replication of different HuNoV strains. Together, these protocols should facilitate the study of HRV and HuNoV in HIEs in many laboratories, and modifications of these basic procedures may be used for replication of other human enteric microbes.

2 Materials

2.1 Maintenance and Differentiation of Three-Dimensional HIEs Derived from Human Small Intestinal Crypts

1. Complete media without growth factors (CMGF−) (*see* Table 1, **Note 1**).
2. Complete media with growth factors (CMGF+) (*see* Table 1, **Note 1**).

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3. DifferentiationMedia: CMGF+ without L-WNT3A, R-spondin, nicotinamide, and SB202190; reduce Noggin to 5 %.
4. Matrigel™, Growth Factor Reduced (GFR), phenol-free: Corning.
5. 1 ml syringe with 25G × 5/8″ needle.
6. 24-well Nunc cell culture-treated multiwell dishes: Thermo Scientific.
7. Accutase: BD Biosciences.
8. Fetal bovine serum: Corning.
9. Allophycocyanin (APC)-conjugated mouse anti-human CD44 antibody (Clone G44-26): BD Biosciences.
10. Round-bottom polystyrene tubes.
11. 300 μM DAPI.

2.2 Rotavirus Infection and Analysis of Differentiated 3D HIEs

1. CMGF−.
2. Differentiation Media.
3. Pancreatin from porcine pancreas: Sigma-Aldrich.
4. Rotavirus stock lysate and appropriate control lysate.
5. 0.22 μm syringe filter.
6. 3 ml syringe.
7. Trypsin: Worthington.
8. Matrigel™, GFR, phenol-free: Corning.
9. LoBind microcentrifuge tubes: Eppendorf.
10. Polyclonal rabbit anti-RV antibody: Laboratory generated [18].
11. Alexa Fluor 488 donkey anti-rabbit antibody: Invitrogen.
12. Cytofix/Cytoperm: BD Biosciences.
13. Perm/Wash buffer: BD Biosciences.

2.3 Establishment of Monolayer HIEs on Transwells and 96-Well Plates

1. CMGF−.
2. CMGF+.
3. Collagen type IV from human placenta: Sigma-Aldrich.
4. Glacial Acetic Acid: Thermo Scientific.
5. 1× DPBS, no calcium, no magnesium: Invitrogen.
6. 0.5 M EDTA.

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7. 0.05 % Trypsin/0.5 mM EDTA: Invitrogen.
8. 5 mM ROCK inhibitor Y-27632: Sigma-Aldrich.
9. Fetal bovine serum: Corning.
10. Transwells: Costar.
11. 40 μm cell strainer.
12. 96-well tissue culture plates.

2.4 Norovirus Infection of Differentiated Monolayer HIEs

1. Human norovirus (HuNoV)-positive and human norovirus-negative stools.
2. Cup horn sonicator (Heat Systems Ultrasonics, Plainview, NY).
3. 5, 1.2, 0.8, 0.45, and 0.22 μm filters (PVDF membrane).
4. Round-bottom 96-well plate.
5. CMGF−.
6. Differentiation Media.
7. Bile (Human, bovine, or porcine): Sigma-Aldrich (*see* **Note 18**).
8. 0.05 % Trypsin-EDTA: Gibco.
9. DMEM: Invitrogen.
10. Fetal bovine serum.
11. Cytofix: BD Biosciences.
12. Stain Buffer containing 5 % BSA: BD Pharmingen.
13. Methanol.
14. PBS.
15. Guinea pig anti-GII.4/Sydney 2012 VP1 antibody: Laboratory generated [12, 21].
16. Alexa Fluor 488 goat anti-guinea pig antibody: Invitrogen.
17. 300 μM DAPI.

3 Methods

3.1 Maintenance and Differentiation of Three-Dimensional HIEs Derived from Human Small Intestinal Crypts

Three-dimensional HIEs were derived from human small intestinal biopsies or surgical specimens following protocols from the Clevers' laboratory and others [14, 15]. In our laboratory, HIEs have been derived from all segments of the small intestine, the colon, and the stomach.

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3.1.1 Reviving Frozen Stocks of HIEs from Liquid Nitrogen

1. *Preparation:* Thaw appropriate amount of Matrigel™ overnight at 4 °C (30 μl/well).
2. Transfer a frozen vial containing HIEs from liquid nitrogen to dry ice.
3. Hold vial under room temperature (RT) tap water until ice detaches from the vial wall.
4. Transfer contents of the vial into a pre-chilled 15 ml conical tube containing 10 ml cold CMGF−.
5. Pellet cells in a swinging bucket rotor centrifuge at 80 × g, 4 °C for 5 min. Remove supernatant.
6. Leave 15 ml tube containing HIE pellet on ice. Resuspend pellet in the appropriate amount of Matrigel™ (30 μl/well) using cold P200 pipette tips. Plate HIEs as droplets in 24-well plates (*see* **Notes 2** and **3**).
7. Let gel solidify for 5–10 min at 37 °C.
8. Add 500 μl of RT CMGF+ to each well and culture in 37 °C incubator with 5 % CO_2.
9. Refresh culture with CMGF+ every other day until the HIEs are ready to be passaged (*see* **Note 4**).

3.1.2 HIE Passage and Differentiation

1. Remove old media from wells leaving the Matrigel™ plug intact.
2. Add 500 μl cold CMGF− to well and mechanically break up Matrigel™ by gently pipetting up and down with a P1000 pipet.
3. Using a 1 ml syringe with a 25G × 5/8″ needle, syringe up and down the contents of each well 2–3 times, then transfer the entire contents into a 15 ml conical tube (multiple wells of the same HIEs can be combined). Add an additional 2× volume of cold CMGF− to dissolve the Matrigel™ (*see* **Note 5**).
4. Pellet cells in a swinging bucket rotor centrifuge at 80 × g, 4 °C for 5 min. Remove supernatant.
5. Plate HIEs with Matrigel™ on 24-well plates as step 6–9 in Sect. 3.1.1.
6. If downstream analysis or infection is desired, switch CMGF+ to Differentiation Media after 4 days in culture for 3–5 days (Fig. 1a). Refresh culture with Differentiation Media every other day until use (*see* **Note 6**).

3.1.3 Flow Cytometry Analysis of Undifferentiated and Differentiated HIEs— The differentiation status of HIEs is critical in HRV and HuNoV infections and can be monitored by many methods including RT-qPCR (some common probes used are listed in Table 2), light microscopy (Fig. 2a), immunofluorescent staining (some common antibodies used are listed in Table 3), and flow cytometry. Here, we describe an easy and quantitative

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method using CD44 staining in flow cytometry. CD44 has previously been shown to be a marker for crypt cells [22].

1. Remove old Differentiation Media from HIE wells, keeping Matrigel™ plug intact.
2. Add 500 μl ice-cold Accutase to each well. Vigorously pipette up and down with a P1000 to dissolve Matrigel™.
3. Incubate at 37 °C for 30 min. Pipette up and down 10 times every 10 min to further break up Matrigel™ and cell clumps.
4. Scrape and transfer all contents into a round-bottom polystyrene tube. Vortex for a final break up of cell clumps.
5. Add 3 ml CMGF− to dilute the Accutase enzyme reaction.
6. If desired, the cell solution can be passed through a 40 μm filter to exclude cell clumps.
7. Pellet cells in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
8. Wash cells with 3 ml CMGF− and pellet cells in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
9. Resuspend cells with 100 μl CMGF− containing 10 % FBS. Add APC-conjugated anti-human CD44 antibody at the manufacturer's recommended concentration. Vortex.
10. Incubate at RT for 30 min or 4 °C for 1 h.
11. Wash with 1 ml CMGF− and pellet cells in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
12. Resuspend cells in 500 μl CMGF− for flow cytometry analysis.
13. If desired, 5 μl of 300 μM DAPI can be added just before analysis. Dead, but not live, cells will incorporate DAPI thus can be excluded during flow analysis.

3.2 Rotavirus Infection and Analysis of Infected 3D HIEs

3.2.1 Rotavirus Infection of Differentiated 3D HIEs

1. *Preparation*: Make rotavirus incubation media by dissolving 0.25 mg/ml pancreatin in Differentiation Media. Allow the pancreatin to dissolve for about 30 min at RT. The undissolved particulates are then filtered out using a 0.22 μm syringe filter with a 3 ml syringe (*see* **Notes 7** and **8**).
2. Activate rotavirus and control stock lysates with 10 μg/ml trypsin at 37 °C for 30 min (*see* **Notes 9** and **10**).
3. Remove old Differentiation Media from wells. Keep Matrigel™ plug intact.
4. Add 500 μl ice-cold CMGF− to each well. Gently pipette up and down with P1000 to dissolve Matrigel™.

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5. Scrape and transfer all contents into a 15 ml conical tube.
6. Add an additional 2× volume of cold CMGF− to dissolve Matrigel™.
7. Pellet in a swinging bucket rotor centrifuge at 80 × g, 4 °C for 5 min. Remove supernatant.
8. Remaining Matrigel™ residue can be seen as a clear clump above the cell clump after centrifugation. Washing steps 6–7 can be repeated until Matrigel™ residue has been completely dissolved.
9. Resuspend HIEs in 200 µl rotavirus incubation media containing the appropriate amount of HRV or control stock lysates. Transfer contents to a LoBind microcentrifuge tube.
10. Incubate at 37 °C, 5 % CO_2 for 1–2 h.
11. Wash unbound virus by adding 1 ml CMGF−.
12. Pellet in a swinging bucket rotor centrifuge at 80 × g, 4 °C for 5 min. Remove supernatant.
13. Repeat washing steps 11–12.
14. Add 500 µl of RT Differentiation Media to each tube and culture infected HIEs in suspension at 37 °C incubator with 5 % CO_2 until analysis.
15. If desired, 1 µg/ml trypsin can be added to differentiation media to allow for multiple rounds of infection. If longer infection duration is desired, 10 µM ROCK inhibitor Y27632 can be added to improve cell survival.

3.2.2 Flow Cytometry Analysis of Rotavirus-Infected 3D HIEs—Flow cytometry analysis measures percentage of rotavirus-infected cells (Fig. 2b, c). Dead cells due to HRV infection will be excluded in this analysis; therefore, this method is better used during early course of infection (up to 24 hpi). If analysis during late course of infection must be monitored, infectivity assays such as fluorescence focus assay and plaque assays can be used. These methods are discussed in Arnold et al. Curr Protoc Microbiology. 2009 [23].

1. Follow steps 1–8 in Sect. 3.1.3 to dissociate 3D HIEs into single cells.
2. Resuspend cells in 250 µl Cytofix/Cytoperm.
3. Incubate at 4 °C for 15–20 min.
4. Wash with 3 ml 1× Perm/Wash buffer. Pellet in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant (*see* **Note 11**).
5. Resuspend in 100 µl 1× Perm/Wash buffer. Add an appropriate amount of polyclonal rabbit anti-RV antibody. Vortex.
6. Incubate at RT for 30 min.
7. Wash with 1 ml 1× Perm/Wash buffer. Pellet in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.

8. Resuspend in 100 µl 1× Perm/Wash buffer. Add Alexa Fluor 488 donkey anti-rabbit antibody at the manufacturer's recommended concentration. Vortex.
9. Incubate at RT for 30 min, protected from light.
10. Wash with 1 ml 1× Perm/Wash buffer. Pellet in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
11. Resuspend in 500 µl 1× Perm/Wash buffer for flow cytometry analysis.

3.3 Establishment of Monolayer HIEs on Transwell and 96-Well Plates

1. *Preparation*: Make 0.5 mM EDTA by diluting 0.5 M EDTA stock in cold 1× DPBS. Prepare 33 µg/ml Collagen IV by diluting the lyophilized powder in 0.6 % acetic acid (*see* **Note 12**).
2. Coat desired number of transwells or 96 wells using 100 µl of cold Collagen IV.
3. Allow Collagen IV to solidify by placing the coated plates at 37 °C for 2 h.
4. Take out the appropriate amount of undifferentiated, fully grown 3D HIEs (*see* **Note 13**).
5. Remove old media from wells leaving the Matrigel™ plug intact.
6. Collect HIEs by adding 500 µl per well of cold 0.5 mM EDTA in DPBS. Scrape and transfer all contents into a 15 ml conical tube.
7. Pellet in a swinging bucket rotor centrifuge at 200 × g, 4 °C for 5 min. Remove supernatant.
8. Dissociate HIEs into single cells by adding 500 µl 0.05 % trypsin/0.5 mM EDTA (see **Note 14**).
9. Incubate at 37 °C for 4 min.
10. Add 1 ml CMGF– containing 10 % FBS to inactivate trypsin.
11. Dissociate HIEs by vigorously pipetting up and down approximately 50 times using a P1000 (*see* **Note 15**).
12. Wet a 40 µm cell strainer with 1 ml CMGF– containing 10 % FBS. Allow the dissociated HIE solution to pass through the 40 µmcell strainer by gravity for 5 s into a 50 ml conical tube to exclude clumps of cells.
13. Pellet filtered cell solution in a swinging bucket rotor centrifuge at 400 × g, RT for 5 min. Remove supernatant.
14. Resuspend cells in the appropriate amount of CMGF+ containing 10 µM Y-27632 (*see* **Note 16**).
15. Take out Collagen IV-coated plates. Remove excess liquid from solidified Collagen IV-coated wells.
16. Plate cell solution on Collagen IV-coated plates, usually 100 µl per well in a transwell plate or 96-well plate (*see* **Note 17**).

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17. If using transwells, add 500 μl RT CMGF+ to the bottom compartment and 100 μl to the upper compartment. If using 96-well tissue culture plates, add 100 μl CMGF+ to each well. Culture in 37 °C incubator with 5 % CO_2.

18. Switch CMGF+ to Differentiation Media the next day. Incubate for 3–5 days refreshing the culture with differentiation media every other day until use (*see* **Note 6**).

3.4 Human Norovirus Infection of Differentiated Monolayer HIEs

3.4.1 Preparation of 10 % HuNoV Stool Filtrates

1. Weigh 0.5 g of HuNoV-positive or HuNoV-negative stool in a 15 ml conical tube. Add 4.5 ml PBS to make a 10 % stool suspension.
2. Break up solids with a 1 ml pipette tip as necessary, then vortex for ~30 s.
3. Keep at RT for 5 min and vortex again.
4. Sonicate using a cup horn sonicator at a setting of 10, 3 times for 1 min, with a 1 min rest on ice between sonications.
5. Centrifuge at 1500 × g for 10 min to remove debris.
6. Collect supernatant in a new 15 ml centrifuge tube and repeat centrifugation one more time.
7. Serially pass the supernatant through 5 μm, then 1.2, 0.8, 0.45, and 0.22 μm filters.
8. Aliquot the resulting 10 % stool filtrate, 100 μl/tube and store at −80 °C.

3.4.2 Norovirus Infection of Monolayer HIEs on 96-Well Plate—Similar protocol can be used for HuNoV infection of transwell HIEs. In our laboratory, infection on monolayer HIEs usually gives a slightly higher yield when compared to transwell HIEs.

1. *Preparation*: Thaw HuNoV-positive and HuNoV-negative stool filtrates at RT. Make 1:10 and 1:100 dilutions of each stool filtrate.
2. In a round-bottom 96-well plate, add 228 μl of CMGF– medium with or without bile (*see* **Note 18**).
3. For each HuNoV-positive and HuNoV-negative stool filtrate, add 12 μl of each diluted sample into the 228 μl CMGF– medium. (This yields 240 μl inocula for infection. 100 μl from each well will be used to inoculate two wells for the 1 hpi and 72 hpi timepoints, respectively.) Each sample should be prepared in triplicate.
4. Wash the HIE monolayers once with ice-cold CMGF−.
5. Gently aspirate the medium from each well.
6. Pipet the inocula up and down 5 times to mix. Transfer 100 μl inocula to the corresponding well containing HIEs. Pipet the inocula on the side of the well, careful not to disturb the HIE monolayer.

7. Incubate the infected plates for 1 h in a 37 °C incubator with 5 % CO_2.
8. Pipet the inocula off each well and gently wash each monolayer three times by applying 100 μl CMGF– medium to the wall of the well and aspirating the wash media off.
9. Add 100 μl of differentiation medium (with or without bile) to each well. The 1 hpi timepoint wells are ready for harvest. The 72 hpi timepoint wells are incubated in a 37 °C incubator with 5 % CO_2 until harvest.

3.4.3 Flow Cytometry Analysis of Norovirus-Infected Monolayer HIEs—Flow cytometry analysis measures percentage of HuNoV-infected cells. Dead cells due to HuNoV infection will not be included in this analysis; therefore, this method is better used during early course of infection (up to 24 hpi). If analysis during late course of infection must be monitored, infectivity assays such as RT-qPCR and fluorescence focus assay are more appropriate. Figure 3a shows sample analysis of HuNoV-infected HIE monolayers using RT-qPCR.

1. Remove old media from well.
2. Add 100 μl 0.05 % trypsin-EDTA.
3. Incubate at 37 °C for 5 min.
4. Add 1 ml DMEM containing 10 % FBS to stop the reaction.
5. Pellet in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
6. Resuspend in 500 μl Cytofix.
7. Incubate at RT for 10 min.
8. Add 1 ml of Stain Buffer and pellet cells in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
9. Resuspend in 900 μl −20 °C methanol.
10. Incubate at 4 °C for 30 min.
11. Wash with 3 ml PBS. Pellet in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
12. Repeat step 11.
13. Resuspend in 100 μl Stain Buffer. Add appropriate amount of guinea pig anti-GII.4/Sydney 2012 VP1 antibody. Vortex.
14. Incubate at RT for 30 min.
15. Wash with 1 ml Stain Buffer. Pellet in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
16. Resuspend in 100 μl Stain Buffer. Add Alexa Fluor 488 goat anti-guinea pig antibody at manufacturer's recommended concentration. Vortex.

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17. Incubate at RT for 30 min. Protected from light.
18. Wash with 1 ml Stain Buffer. Pellet in a swinging bucket rotor centrifuge at 400 × g, 4 °C for 5 min. Remove supernatant.
19. Resuspend in 500 μl Stain Buffer for flow cytometry analysis.

3.4.4 Immunofluorescent Staining of Human Norovirus-Infected Monolayer HIEs—

Immunofluorescent staining can help identify and locate the HuNoV-infected cells. Other antibodies can be combined to examine other structural and nonstructural viral proteins and/or cellular components.

1. Remove old media from well.
2. Wash with 100 μl PBS, then aspirate off wash media.
3. Add 100 μl ice-cold 100 % methanol to fix. Incubate at −20 °C for 20 min. Aspirate off fixative.
4. Wash with 100 μl PBS, then aspirate off wash media. Repeat twice.
5. Add 100 μl PBS containing 5 % FBS to block nonspecific binding of antibodies. Incubate at RT for 30 min. Aspirate off blocking buffer.
6. Add 100 μl PBS containing 5 % FBS and appropriate amount of guinea pig anti-GII.4/Sydney 2012 VP1 antibody.
7. Incubate at RT for 2 h or at 4 °C overnight.
8. Wash with 100 μl PBS, then aspirate off wash media. Repeat twice.
9. Add 100 μl PBS containing 5 % FBS and appropriate amount of Alexa Fluor 488 goat anti-guinea pig antibody at manufacturer's recommended concentration.
10. Incubate at RT for 1 h protected from light.
11. Wash with 100 μl PBS, then aspirate off wash media. Repeat twice.
12. Add 100 μl PBS containing 300 nM DAPI.
13. Incubate at RT for 1 min.
14. Wash with 100 μl PBS, then aspirate off wash media. Repeat twice.
15. Add 100 μl PBS and visualize under immunofluorescent microscope (Fig. 3b).

4 Notes

1. L-WNT3a cell line is commercially available from ATCC. R-Spondin 1 cell line is from Trevigen. Noggin cell line is a generous gift from Dr. Gijs van den Brink (University of Amsterdam). Conditioned media from L-WNT3a, R-Spondin 1, and Noggin can be aliquoted and stored at −20 °C for 1 month. Avoid freeze and thaw cycles. CMGF+ can be kept at 4 °C for up to 2 weeks. CMGF− and Differentiation Media can be kept at 4 °C for up to 1 month.

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2. Matrigel™ is liquid at 4 °C and quickly solidifies at warmer temperature; therefore, all tubes and pipette tips must be pre-chilled at 4 °C before plating and kept on ice during plating.
3. Our frozen stock usually contains two wells of fully grown HIEs. Each frozen stock is plated onto four wells during revival.
4. Depending on plating density, HIEs are usually ready to be passaged after 6–7 days of growth. A 1:5 ratio can be used as a starting point for passaging. It is important to passage HIEs a few times after revival before using for experiments.
5. The number of syringing must be experimentally determined. Depending on an individual's force and the number of days before the next passage, HIEs can be broken up into desired sizes.
6. The length of HIE differentiation must be experimentally determined for each HIE line and viral infection. Typical differentiation will change HIEs from a shining finish with thin epithelial lining to a multi-lobular structure with thickened epithelium (Fig. 2a), although variability is observed depending on the intestinal segment and tissue source.
7. Pancreatin is difficult to dissolve in aqueous solution. To ensure pancreatin is maximally dissolved, the solution should be vigorously vortexed and incubated at RT for at least 30 min.
8. Pancreatin is easily degraded. Make up fresh pancreatin solution every time.
9. For generating and quantifying rotavirus stocks, please refer to Arnold et al. *Curr Protoc Microbiology*. 2009 [23].
10. Rotavirus incubation with trypsin is important for the cleavage of the cell attachment protein VP4. This step is crucial for successful infection.
11. Fixed cells can be kept in 1× Perm/Wash buffer at 4 °C for up to 1 week.
12. Collagen IV solution can be aliquoted and stored at a stock concentration of 1 mg/ml at −20 °C for 1 year. Avoid freeze and thaw cycles. Dilute 1 mg/ml stock solution by 1:30 using cold H_2O before use. Collagen solution will solidify at RT. Keep cold on ice bath.
13. 3D HIEs should be in culture for 7 days before being used for monolayer plating. Each 3D HIE well should be examined under light microscope to ensure approximately 100 live, cystic HIEs. Dead HIEs will look small, dark, and rounded. In our laboratory, approximately one well of 3D HIEs can be used to plate 1 transwell or 2.5 wells on a 96-well tissue culture plate.
14. If pooling more than five wells of 3D HIEs in the same tube, the volume of 0.05 % trypsin/0.5 mM EDTA should be increased to 1 ml per well to ensure dissociation of all HIEs. We do not recommend pooling more than ten wells per tube for this dissociation process.

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15. Avoid bubbles during dissociation by pipetting against the sides of a 15 ml conical tube. Vigorous pipetting is absolutely necessary to ensure single-cell dissociation from 3D HIEs. This is also crucial to ensure all cells are passed through a 40 μm cell strainer at the later step.

16. Y-27632 inhibits Rho-associated, coiled-coil-containing protein kinase 1 and 2 (ROCK 1 and 2). Addition of Y-27632 enhances survival of dissociated single cells by preventing apoptosis.

17. HIE cell solutions should be added drop-wise onto the center of the monolayer or transwell. Cells will spread out and coat the transwell. Do not rotate or swirl the seeded plates.

18. Bile can be freshly isolated or lyophilized then reconstituted. Bovine and porcine bile is commercially available from Sigma-Aldrich. The percentage of bile used in HuNoV Incubation Media must be experimentally determined to maximize infection and minimize cell death for each virus strain and HIE line, respectively. In our hands, 5 % human bile and 0.03 % bovine bile gave the most optimal replication for GII.3 HuNoV. GII.4 HuNoV does not require bile for replication, but the presence of bile enhances replication [12].

Acknowledgments

The techniques described in this article were developed by research on rotaviruses and noroviruses that was supported by National Institutes of Health (NIH) Grants U19-AI116497, R01 AI080656, PO1 AI 057788 (to Mary K. Estes), U18-TR000552 (to Mark Donowitz), P30 DK-56338 (to Hashem El-Serag), F30 DK107173 (to Winnie Zou), Agriculture and Food Research Initiative competitive grant 2011-68003-30395 from the USDA National Institute of Food and Agriculture (to LeAnn Jaykus), and Howard Hughes Medical Institute Grant 570076890 (to Kapil Saxena).

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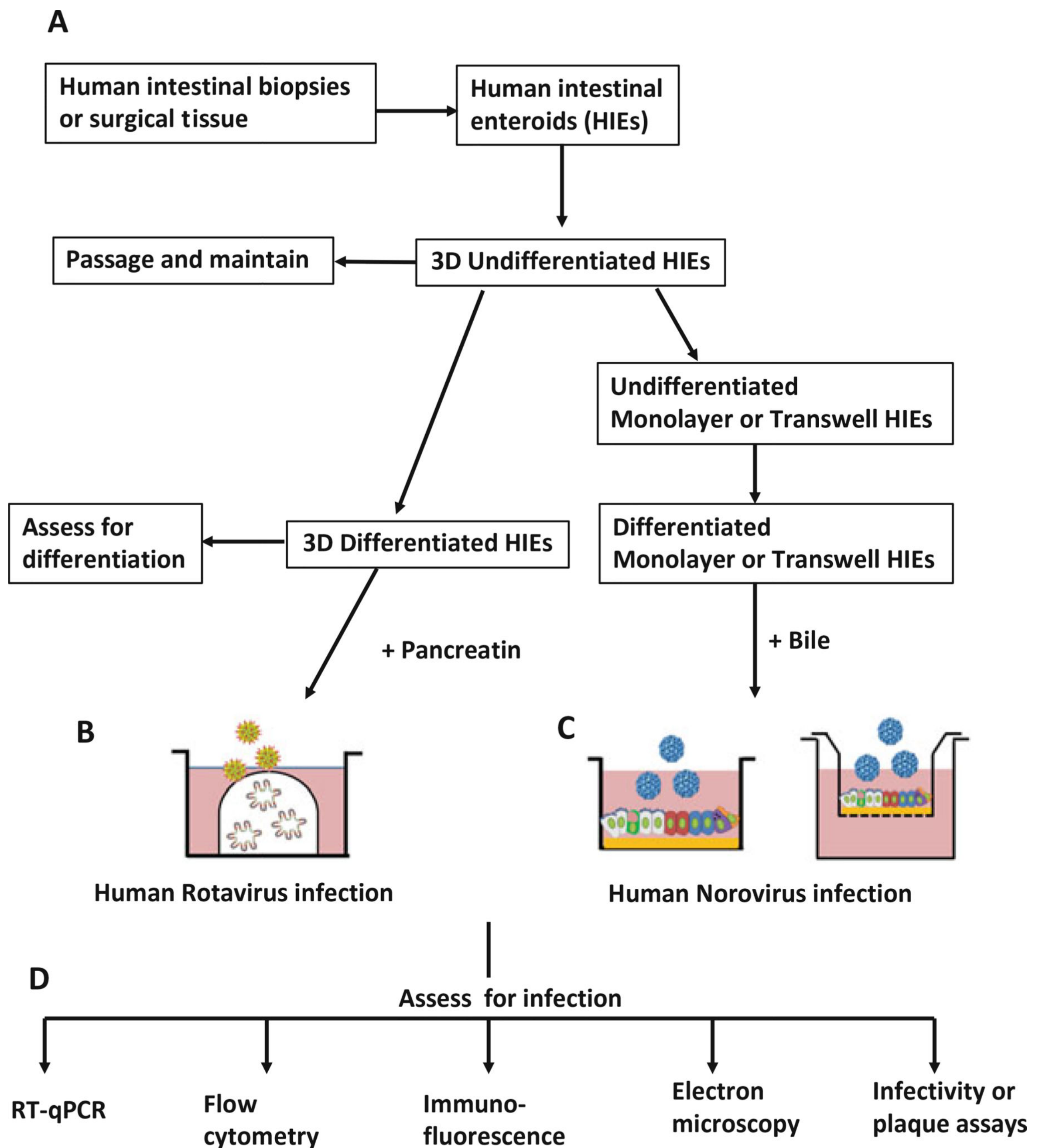

Fig. 1.

Human rotavirus and norovirus infections in human intestinal enteroids (HIEs). (**a**) HIEs can be revived and passaged from frozen stocks and maintained in undifferentiated format. Differentiation is required for both rotavirus and norovirus infections. (**b**) Human rotavirus infection uses 3D HIEs in the presence of pancreatin. (**c**) Human norovirus infection uses monolayer or transwell HIEs in the presence of human, bovine, or porcine bile. (**d**) Infection can be monitored using various methods

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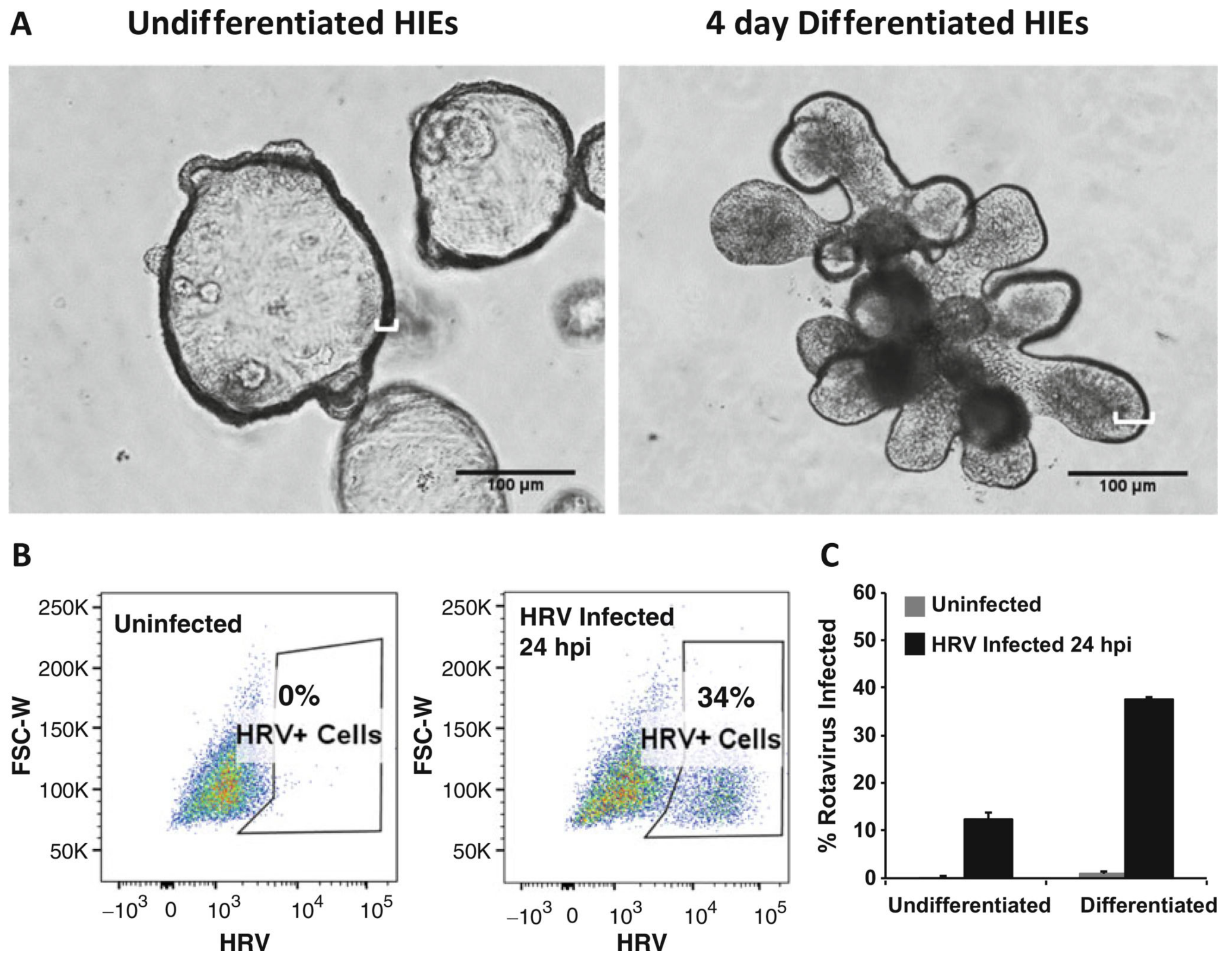

Fig. 2.

Differentiation of human intestinal enteroids (HIEs). (**a**) Undifferentiated HIEs appear cystic and shining, whereas differentiated HIEs appear multi-lobular, with a thickened epithelium. White brackets denote epithelial lining. Note that differentiated HIEs can also often be circular depending on the intestinal segment and tissue origin. (**b**) Representative scheme for flow cytometry analysis of human rotavirus (HRV) infection. Single, live cells are gated using forward and side scatters. HRV+ cells can then be detected in the infected sample. (**c**) Differentiation of HIEs is required for HRV infection. Percentage of HRV-infected cells assessed by flow cytometry. $N = 3$ jejunal HIEs

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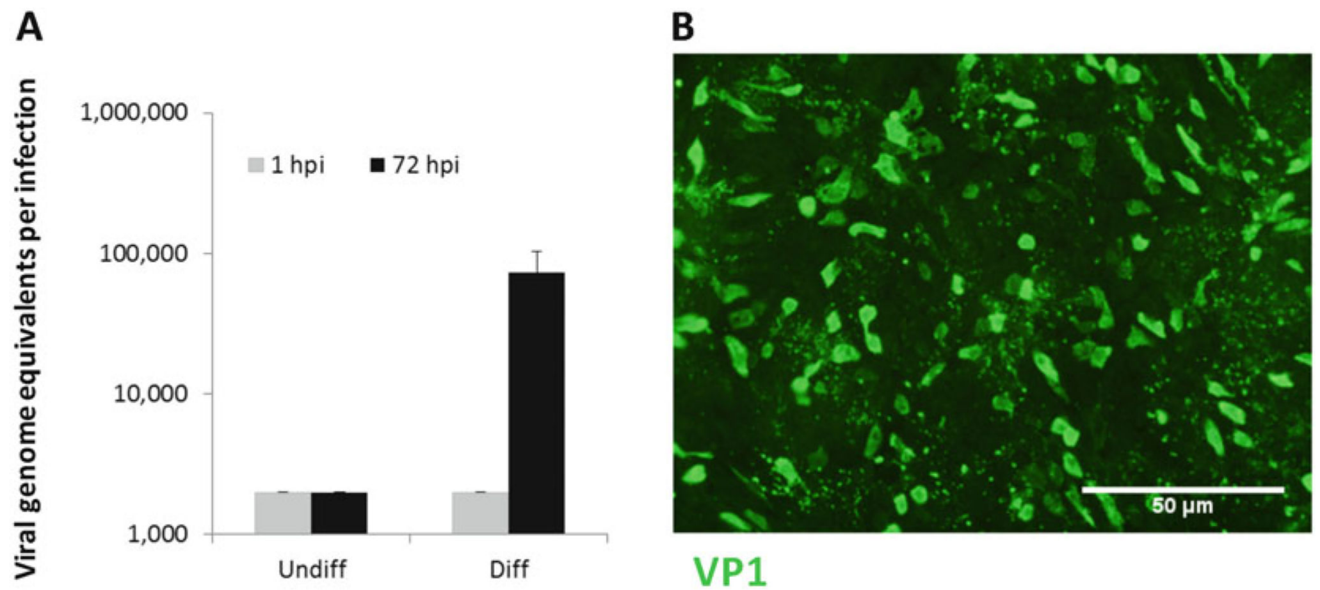

Fig. 3.

Differentiation allows for human norovirus (HuNoV) infection. (**a**) Viral genome equivalents per infection in undifferentiated and differentiated jejunal HIE monolayers, assessed by RT-qPCR comparing 1 to 72 hpi. $N = 3$ jejunal HIE infections. (**b**) Expression of viral capsid protein VP1 in jejunal HIE monolayers detected using anti-VP1 antibody in immunofluorescent staining. Culture was innoculated with 9×10^7 HuNoV genome equivalents

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Table 1

Composition of complete media for HIE maintenance and differentiation

Reagent	Final concentration	Origin
Complete media without growth factors (CMGF−)		
Advanced DMDM/F12	N/A	Invitrogen
GlutaMAX-1	2 mM	Invitrogen
HEPES	10 mM	Invitrogen
Penicillin/streptomycin	100 U/ml	Invitrogen
Complete media with growth factors (CMGF+)		
CMGF−	N/A	N/A
L-WNT3A-conditioned media	50 %	ATCC
R-Spondin-conditioned media	20 %	Trevigen
Noggin-conditioned media	10 %	A gift from Dr. Gijs van den Brink (University of Amsterdam)
B27	1×	Invitrogen
N2	1×	Invitrogen
N-acetylcysteine	1 mM	Sigma-Aldrich
Mouse recombinant EGF	50 ng/ml	Invitrogen
[Leu15]-Gastrin I	10 nM	Sigma-Aldrich
Nicotinamide	10 mM	Sigma-Aldrich
A-83-01	500 nM	Tocris
SB202190	10 μM	Sigma-Aldrich

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Table 2

RT-qPCR probes used to analyze differentiation status of HIEs

Marker	Changes during differentiation	Cat. No. (Life Technologies)
CD44	Down	Hs01075861_m1
LGR5	Down	Hs00969422_m1
MUC2	Up	Hs03005103_g1
PCNA	Down	Hs00427214_g1
Sucrase isomaltase	Up	Hs00356112_m1

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Table 3

Antibodies used to analyze differentiation status of HIEs

Marker	Cell type marked	Company (concentration)
Sucrase isomaltase	Enterocytes	Santa Cruz (1:100)
Muc2	Goblet cells	Santa Cruz (1:500)
Chromagranin A	Enteroendocrine cells	Novus Biologicals (1:100)
UEA-1 lectin	Histo-blood group antigens	Sigma-Aldrich (1:500)
E-cadherin	Adherens junctions	BD Biosciences (1:100)
Lysozyme	Paneth cells	Dako (1:100)