PASSAGING HUMAN INTESTINAL ORGANOIDS AND MONOLAYERS SET UP

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Method Article

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Abstract

Human intestinal enteroids (HIE) have been found to support enteric virus replication, which is especially relevant for studying human rotavirus and human norovirus infections. Thus, the HIE system is being used as a biologically significant human system to assess the efficiency of preventive and control strategies to prevent infection, which is relevant in clinical, therapeutics, and food safety fields. This protocol is intended to be used for the maintenance of human intestinal enteroids (HIE) (Procedure A) and set up of single cell monolayers to be differentiated and infected for viral replication (Procedure B). The procedures described are based on the original publications on human rotavirus and human norovirus replication in HIE (Ettayebi et al., 2016; Saxena et al., 2015; Zou et al., 2019), with the modifications implemented elsewhere (Ettayebi et al., 2021) and at VISAFElab for making the routinely handling of the system more practical. The main novel implementation refers to the use of the gentle cell dissociation reagent (produced by Stem Cell Technologies) to dissociate HIE domes for routine passaging or for producing single-cell suspensions for monolayers set up. Those modifications have been proven to support the human norovirus and human rotavirus replication features originally described for the HIE system.

Introduction

Reagents

Reagent

IntestiCult OGM Human Kit, STEM CELL, Cat. 6010

Y-27632 dihydrochloride, Sigma-Aldrich, Cat. Y0503

Collagen from Human Placenta, Sigma-Aldrich, Cat. C5533

HEPEs 1M, Gibco, Cat. 12509079

Cell strainer 40µm, Biologix, Cat. 15-1040

Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Corning, Cat. 356231

GlutaMAX-I, Gibco, Cat. 13462629

Advanced DMEM/F12, Gibco, Cat. 11540446

Penicillin/Streptomycin, Gibco, Cat. 11548876

Gentle Cell Dissociation Reagent, STEM CELL, Cat. 100-0485

0.05% Trypsin-EDTA, Gibco, Cat. 11580626
Reagent preparations

**IntestiCult™ Organoid Growth Medium (Human)**

The IntestiCult OGM Human Kit is composed by IntestiCult™ OGM Human Basal Medium (Component A) and the Organoid Supplement (Component B). Thaw and prepare 5-10 ml aliquots to avoid freezing cycles.

Organoid Growth Medium (OGM): mix in equal parts (1:1) Component A and Component B.

**Organoid Differentiation Medium (ODM)**

Organoid Differentiation Medium (ODM): mix in equal parts (1:1) Component A and CGMF-.

**Complete media without growth factor (CGMF-)**

- Advanced DMEM/F12, 48.5 mL
- Penicillin/Streptomycin 100x, 0.5 mL
- GlutaMAX-I 100x, 0.5 mL
- HEPES 1M 0.5 mL

Equipment

Procedure

**Procedure A. Passaging of human intestinal organoids.**

1. Warm a 24-well tissue culture-treated plate in a 37°C incubator for at least 2 hours.

2. Prepare IntestiCult™ Organoid Growth Medium (Human) (ref #06010, STEMCELL) mixing Component A (Basal Medium) and Component B (Supplement). Add 2 μL of 5 mM Y-27632 per milliter of Organoid Growth Medium (10 μM final concentration). Mix thoroughly and warm to room temperature (15 - 25°C).
NOTE: For each well to be passaged, 750 μL of medium will be required. 3 mL of medium is sufficient for 4 culture domes. If preparing a different number of culture domes, adjust volume of medium accordingly.

3 mL IntestiCult™ Organoid Growth Medium = 1.5 mL Component A + 1.5 mL Component B + 6 μL of 5 mM Y-27632

3. Thaw Matrigel® on ice; for each well to be plated, ≈35 μL of Matrigel® will be required.

4. Place CGMF- supplemented with 10% FBS on ice.

5. Carefully remove and discard medium from each well to be passaged, without disturbing the Matrigel® dome.

6. Add 500-750 μL of room temperature (15 - 25°C) Gentle Cell Dissociation Reagent (GCDR) on top of the exposed dome in each well.

7. Pre-wet a 1 mL pipette tip with GCDR; use this pipette tip to thoroughly scrape the Matrigel® dome free of the well floor. Pipette the GCDR in the well up and down 2 - 3 times to break up the dome and the organoids. Ensure all pieces of Matrigel® have been rinsed free of the plate.

NOTE: When pipetting up and down, avoid touching the bottom of the well with the pipette tip.

8. Using the same pipette tip, transfer the organoid mixture to a 15 mL conical tube.

9. Add 500-750 μL of GCDR to the newly emptied well. Using a pipette tip pre-wetted with GCDR, pipette the GCDR up and down 2 - 3 times to rinse the well. Transfer the contents of the well to the 15 mL conical tube from step 8.

10. Repeat steps 7 - 9 for each well to be passaged.

11. Incubate the tubes at room temperature (15 - 25°C) on a rocking platform set at medium speed (~40 rpm) for 10 minutes.

12. Centrifuge the tubes at 290 x g for 5 minutes at 2 - 8°C. Gently pour off and discard the supernatant.

13. Add 1 mL of ice-cold CGMF- + 10% FBS to each tube. Using a pre-wetted 1 mL pipette tip, resuspend organoids by pipetting up and down vigorously 15 times.

NOTE: Avoid touching the side/bottom of the tube with the pipette tip.

14. Centrifuge the sample at 200 x g for 5 minutes.

NOTE: The following steps are for plating 50 μL culture domes giving a 2:1 final mixture of Matrigel® and cell suspension in Organoid Growth Medium (e.g. for 8 x 50 μL culture domes, add 266 μL Matrigel® and 133 μL cell suspension in Organoid Growth Medium to the sample tube).
15. Remove the 24-well plate from the 37°C incubator.

16. Aspirate as much as possible of supernatant and adjust the volume of the cell suspension to desired volume (e.g. 133 μL) using Organoid Growth Medium. Resuspend the pellet by gentle pipetting.

17. Pre-wet a 1000 μL pipette tip with Organoid Growth Medium. Add 266 μL of Matrigel® to the sample tube. Pipette up and down 10 times to thoroughly resuspend the pellet. Avoid introducing bubbles.

18. Using a pre-wetted 100 μL pipette tip, draw up 50 μL of the Matrigel®-crypt suspension and add to 1 of the 8 central wells of a 24-well tissue culture-treated plate as follows:
   a. Hold the pipette vertically over the center of the well. Bring the pipette tip near to but not in contact with the floor of the well.
   b. Slightly depress the plunger until a droplet is visible on the end of the pipette tip.
   c. Slowly lower the pipette until the droplet touches the floor of the well.
   d. Gently dispense (only to the first stop on the pipette) the remaining volume while lifting the pipette away from the well.

   NOTE: Work quickly to plate the Matrigel®-crypt suspension within ~60 seconds of removing it from ice.

19. Repeat step 5 until all of the Matrigel®-crypt suspension is dispensed.

20. Carefully transfer the plate to a 37°C incubator. Incubate at 37°C for 10 minutes to allow domes to solidify. Do not disturb the domes.

21. Add 750 μL of IntestiCult™ Organoid Growth Medium + Y-27632 to each well by pipetting the medium gently down the wall of the well. Do not pipette directly onto the domes.

22. Add sterile PBS to unused wells and incubate at 37°C and 5% CO₂.

23. Perform a full medium change with IntestiCult™ Organoid Growth Medium (Y-27632 is not required) every other day. Once a week medium replacement can be performed after 3 days (e.g., on Friday and on Monday).

Procedure B. SET UP OF HIE SINGLE CELL MONOLAYERS.

Use one 7 days old passage 3D well (with good HIEs and good density) to 3-5 96 wells plate (usually on Wednesday). Two days after (Friday), remove OGM, replace with 100 μl ODM. The monolayers should be
100% confluent and differentiated after 4-5 days (Tuesday or Wednesday).

1. Add 100 µL collagen Type IV (1:30 in H2O, Sigma-Aldrich, ref. C5533) in each well designed for monolayer. Dispense the collagen directly in the center of each well and gently shake to evenly distribute. Plates must be coated the day of use. Transfer plates to 37°C incubator for at least one hour.

2. Remove all medium from the organoid cultures without disturbing the organoids within the Matrigel domes.

3. Add 500-750 µL of Gentle Cell Dissociation Reagent (GCDR) to each well of organoids to be harvested. Using a 1 mL tip, vigorously pipette up and down to disrupt the Matrigel® dome and resuspend the organoids. Pool the harvested organoids (up to 8 wells) in a 15 mL conical tube.

4. Add 500-750 µL of GCDR to the newly emptied well. Using a pipette tip pre-wetted with GCDR, pipette the GCDR up and down 2 - 3 times to rinse the well. Transfer the contents of the well to the 15 mL conical tube from step 2.

5. Incubate at room temperature for 10 minutes with gentle agitation or rocking (40 rpm).

6. Centrifuge at 200 - 300 x g for 5 minutes at 2 - 8°C.

7. Remove and discard the supernatant. Add 5 mL ice-cold DMEM/F-12 to resuspend organoids. Centrifuge at 200 x g for 5 minutes at 2 - 8°C.

8. Aspirate supernatant, removing as much as possible, being careful not to disturb the pellet. Add 500 µL (up to 3-4 3D wells collected) or 1 mL (> 4 wells) of warm (37°C) Trypsin-EDTA (0.05%) to resuspend organoids.

9. Incubate for 4 min at 37°C and then add 1 ml of DMEM/F-12 with 10% FCS to inactivate the trypsin.

10. Dissociate the enteroids by vigorously pipetting up and down for ~50 times using a P1000 (avoid making bubbles). Pipet against the side of the 15 ml tube to avoid making bubbles and to dissociate the cells.

11. Wet the cell strainer (40 µm) with 1 ml of CMGF- containing 10% FBS. Take the cap off of a 50 ml centrifuge tube and put the cell strainer on top of the tube. Using a P1000, add the cells to the top of the cell strainer. The cells will pass through the cell strainer by gravity flow. Rinse the walls of the tube with 1ml of CMGF- containing 10% FBS and pass it through the cell strainer. Discard the cell strainer containing cell clumps.

12. Pellet the cells that passed through the cell strainer at 200 x g in a swinging bucket rotor for 5 minutes at 2 - 8°C.
13. Remove the collagen from the 96 wells plate.

14. Discard as much as possible of the supernatant and resuspend the single cell enteroid pellet in the appropriate amount of OGM containing 10 µM Y-27632. Usually the ratio 1:3-5 should be used depending on the density of initial 3D HIEs. Plate 100 µl / well.

15. Two days after plating (usually on Friday), remove OGM, replace with 100 µl ODM.

Troubleshooting

Time Taken

Procedure A takes around 2 hours for 8-16 3D HIE wells to be simultaneously passaged. If higher number of domes will be passaged, it is recommended to process them separately.

Procedure B takes 1.5-2 hours. It is recommended to coat the wells with collagen at first, so they can be incubated while the operator proceeds with the protocol.

Anticipated Results

References


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Supplementary Files

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