INTRODUCTION

The intestine is the fastest self-renewing tissue in mammals with a self-renewal rate of about 3-5 days. IEC proliferation and differentiation are driven by intestinal epithelial stem cells (IESCs) located at the base of crypts, which are either active or quiescent.1,2 Active IESCs, the majority of which are Lgr5+ crypt base columnar cells, maintain lineage development and self-renewal with rapid cycling.3 They are highly sensitive to intestinal injury.3 In contrast, quiescent IESCs are present at the “+4” crypt position, and contribute to regeneration, particularly during recovery from injury.4 Interestingly, quiescent IESCs are also labeled as a Lgr5+Ki67-, Bmi1- or Lrig1+ population that can be reactivated to Lgr5hi IESCs.5-8 These studies indicate that IESCs retain high plasticity during lineage regeneration. IESCs asymmetrically divide into one new IESC and one transient amplifying (TA) cell to maintain intestinal homeostasis, or divide symmetrically into two progenitors in the TA zone only upon tissue expansion or damage.9

IESC progenitors can be differentiated into five major types of IEC lineages: enterocytes, goblet cells, enteroendocrine cells, tuft cells, and/or Paneth cells in the colon or small intestines. Disassociated crypts or single Lgr5+ IESCs can be used to produce crypt units in vitro, which are called organoids. Thus, enteroids from intestine and colonoids from colon are the ideal tools for studying IESC self-renewal and differentiation. We have employed the crypts from Lgr5-EGFP-IRES-creERT2 reporter mouse line to grow Lgr5+ enteroids.10 In this article, we summarize the procedure of organoid culture derived from single Lgr5+ cells, and provide a simple and practical way to examine their viability and functions. An overview of the experimental workflow is given in Figure 1.

MATERIALS

For these experiments, all animal studies were approved by the Institutional Animal Care and Use Committee (IACUA, HXN16002).
2.1 | Mice

LGR5 reporter mice (Lgr5-EGFP-IREs-creERT2) were purchased from Jackson Lab, Bar Harbor, MA (stock no. 008875).

2.2 | Reagents

Matrigel was purchased from Thermo Fisher (Waltham, MA). EGF, Noggin, R-spondin, and Y-27632 were purchased from R&D.
Organoid Growth Medium was purchased from STEMCELL Technologies (Vancouver, Canada). Antibodies specific for Lysozyme (Cat no. ab108508), DCLK1 (Cat no. ab31704), and Chromogranin A (Cat no. ab15160) used for immunofluorescent staining were purchased from Abcam (Cambridge, MA); antibodies specific for E-cadherin (Cat no. 3195) were purchased from Cell Signaling Technology (Boston, MA). All other reagents including ethylenediaminetetraacetic acid (EDTA), Dulbecco's phosphate-buffered saline without Ca²⁺ and Mg²⁺ (DPBS), methylene and Blocking Buffer (1× PBS/5% normal serum/0.3% Triton™ X-100) were purchased from Sigma (St. Louis, MO).

### 3 METHODS AND RESULTS

#### 3.1 Single Lgr5⁺ cell sorting and culture of organoid

1. Thaw the Matrigel overnight in a refrigerator at 4°C and preincubate a four-well plate in a CO₂ incubator at 37°C. Note: Matrigel can be easily polymerized at room temperature, Therefore, all tubes and pipette tips should be equilibrated to 4°C before the addition of Matrigel. The prewarmed plate allows the Matrigel to polymerize quickly and make the Matrigel droplet small in diameter.

2. Euthanize mice with CO₂ as approved by the Institutional Animal Care and Use Committee (IACUA, HXN16002). Sterilize the abdomen of the mouse with 70% ethanol and place the ventral side up.

3. Open the abdominal cavity to dissect intestine on ice.

4. Flush fecal pellets out of the intestine using a 10-mL syringe with ice-cold DPBS. The intestine is inverted with a catheter and dissected into pieces with 2 × 2 mm² size, then vigorously washed on a shaking water bath to remove any remaining fecal matter.

5. The tissue fragments are incubated in 5 mmol/L EDTA for 20 minutes on ice during which the pieces of intestines are pipetted up and down every 10 minutes and monitored for the release of crypts under the microscope. Shake manually 4-8 minutes depending on the tissue type. Check for crypt morphology under a fluorescence microscope and stop shaking when 70% of the crypts are released (Figure 2A,B). Note: Shake the tube by hand with a back and forth motion for two cycles per second. Vigorous shaking might damage the crypts and hence should be avoided.

6. Filter the supernatant through a 70 μmol/L pore size filter for intestinal crypts or 100 μmol/L filter for colonic crypts to exclude the debris and collect the live crypt fraction into a 50-mL conical tube.

7. Isolated crypts are spun down at 250 g for 10 minutes at 4°C.

8. Resuspend the pellet in 2 mL ice-cold DPBS; the crypt suspensions are dissociated to individual cells with a needle (G26).

9. Dissociated cells are passed through a cell strainer with a pore size of 20 μmol/L to prevent clogging of the FACS instrument. Count dissociated cells under a microscope and resuspend 2 × 10⁷ cells in a polypropylene tube with 2 mL organoid growth medium.

10. IESCs are isolated as Lgr5 GFPʰⁱ with a BD FACS Aria II cell sorter. Lgr5 GFPʰⁱ, GFPʰ𝑤 and GFP⁻ IESCs are identified by their endogenous GFP expression and gated by forward scatter, side scatter, and pulse-width parameter (FSC-A vs FSC-H and SSC-W vs SSC-H). The green autofluorescence can be excluded by plotting the GFP channel against the phycoerythrin channel. (Figure 2C).

11. Sorted Lgr5 GFPʰⁱ cells are placed into culture medium containing Y-27632 (10 mmol/L).

12. Centrifuge the sorted cells 10 minutes at 500 g, 4°C. Gently remove the supernatant as much as possible.

13. Add all growth factors into the Matrigel: R-spondin 1 (1 μg/mL), Noggin (100 ng/mL), EGF (50 ng/mL), and Wnt-3a (2.5 ng/mL).

14. Use a chilled pipette tip to mix sorted cells (~2500) with 50 μL mixed Matrigel and slowly place it in the center of the prewarmed plate. Note: Rapidly pipette up and down to mix the cells and Matrigel to avoid air bubbles.

15. Place the four-well plate in a 37°C, 5% CO₂ incubator for 20 minutes to allow complete solidification of the Matrigel. Note: Make sure the Matrigel solidifies before adding the medium to avoid Matrigel dissolution.

16. Overlay Matrigel with 500 μL organoid growth medium.

17. Every 4 days, replace the medium with fresh complete minigut medium.

18. Enteroids are derived from the small intestine and those from the colon are colonoids. Monitor organoid formation and growth rate each day by microscopy (Figure 2D,E).
3.2 Lineage identification in organoids

1. Remove medium and add 500 μL freshly prepared 4% para-formaldehyde (PFA) to each well overnight at 4°C.
2. Remove the PFA and add 1 mL ice-cold DPBS, then break the Matrigel with 1000-μL pipette tip in a microcentrifuge tube.
3. Centrifuge the organoids for 10 min at 200 g, 4°C and gently discard the supernatant.
4. Add 10 μL 2% methylene blue to the microcentrifuge tube and leave for 20 minutes at room temperature. Note: Methylene blue staining is to visualize the organoid in OCT.
5. Resuspend organoids in 1000 μL DPBS and centrifuge for 10 minutes at 200 g and gently discard the supernatant.
6. Embed organoids in OCT compound with a 200 μL pipette tip and section into 4-5 mmol/L sections on plus slides. Note: When using the 200 μL tip to pipette the Matrigel, it is advised to cut the tip at the tapered end and coat the tip with FBS to avoid loss of organoid.
7. Rinse the slides in 75% ethanol for 5 minutes to remove methylene blue.
8. Heat slides in a microwave submersed in 10 mmol/L sodium citrate buffer at pH 6.0 until boiling is initiated; follow with 15 minutes at a sub-boiling temperature. Cool slides for 10 minutes and then wash the slides with PBST.
9. Add 100 μL 0.1% Triton X-100 and leave for 30 minutes and then wash slides with PBST. Note: Triton X-100 is the most popular detergent for improving the penetration of the antibody. However, it is not appropriate for membrane-associated antigens.
10. Add 100 μL blocking buffer to each slide and leave for 30 minutes.
11. Remove blocking solution and add 100 μL primary antibody lysozyme (4 μg/mL), Dclk1 (1 μg/mL), Chromogranin A (CHGA, 10 μg/mL), and E-cadherin (5 μg/mL). Incubate overnight at 4°C.
12. Decant the primary antibody solution and wash the slide in PBST.
13. Incubate the slide with a second antibody for 1 hour at room temperature in the dark.
14. Coverslip slides with DAPI.
15. Take an image with Leica DMi8 (DFC9000GT) (see Figure 3).

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REFERENCES

1. Barker N, van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;449:1003-1007.
2. Sangiorgi E, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet. 2008;40:915-920.
3. Tian H, Biels H, Warming S, et al. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. Nature. 2011;478:255-259.
4. Takeda N, Jain R, LeBoeuf MR, Wang Q, Lu MM, Epstein JA. Interconversion between intestinal stem cell populations in distinct niches. Science. 2011;334:1420-1424.
5. Yan KS, Gevaert O, Zheng GXY, et al. Intestinal enteroendocrine lineage cells possess homeostatic and injury-inducible stem cell activity. Cell Stem Cell. 2017;21:78-90.e6.
6. Basak O, Beumer J, Wiebrands K, Seno H, van Oudenaarden A, Clevers H. Induced quiescence of Lgr5+ stem cells in intestinal organoids enables differentiation of hormone-producing enteroendocrine cells. Cell Stem Cell. 2017;20:177-190.e4.
7. Basak O, van de Born M, Korving J, et al. Mapping early fate determination in Lgr5+ crypt stem cells using a novel Ki67-RFP allele. EMBO J. 2014;33:2057-2068.
8. Powell AE, Wang Y, Li Y, et al. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. Cell. 2012;149:146-158.
9. Snippert HJ, van der Flier LG, Sato T, et al. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5+ stem cells. Cell. 2010;143:134-144.
10. Gilbert S, Nivarthi H, Mayhew CN, et al. Activated STAT5 confers resistance to intestinal injury by increasing intestinal stem cell proliferation and regeneration. Stem Cell Rep. 2015;4:209-225.
11. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5+ stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature. 2009;459:262-265.