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TECHNICAL NOTE

Lgr5$^+$ intestinal stem cell sorting and organoid culture

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Abstract

Intestinal epithelial stem cells (IESCs) are one of the most rapidly self-renewing and proliferating adult stem cells. The IESCs reside at the bottom of intestinal and colonic crypts, giving rise to all intestinal epithelial lineages and maintaining intestinal epithelial replenishment. The technique of three-dimensional culture based upon intestinal stem cell biology has been recently developed to study gastrointestinal development and disease pathogenesis. Here, we summarize the techniques used to isolate Lgr5-positive IESCs to form the enteroids from intestine or colonoids from colon, and present the means to examine these organoid functions. This study will provide a simple and practical way for producing intestinal tissues in the laboratory.

KEYWORDS
colonoids, enteroids, Lgr5 intestinal stem cells, organoid culture

1 | 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.[1] 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^{low}Ki67$^+$, Bmi1$^+$ or Lrig1$^+$ population that can be reactivated to Lgr5^{hi} 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 way to examine their viability and functions. An overview of the experimental workflow is given in Figure 1.

2 | MATERIALS

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

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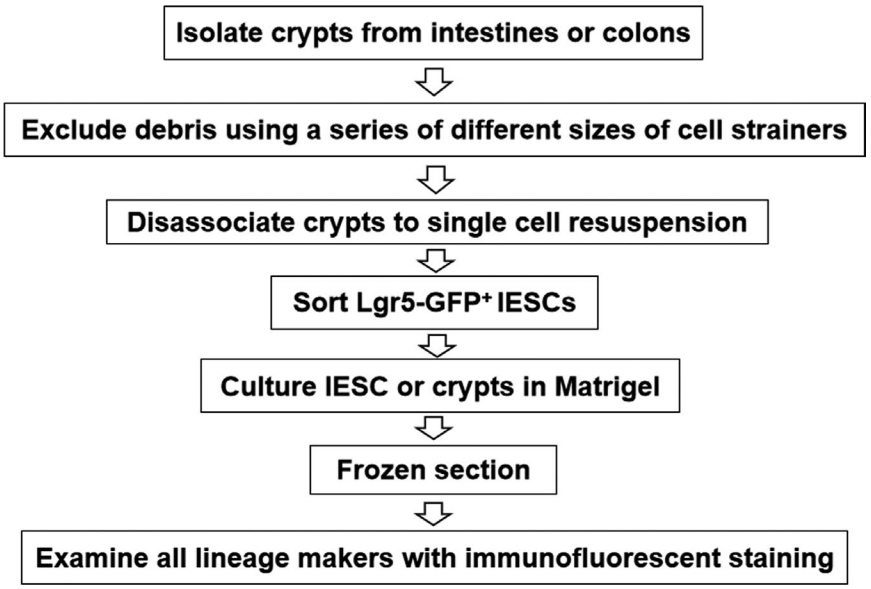

FIGURE 1 Flowchart of Lgr5 IESC culture and staining

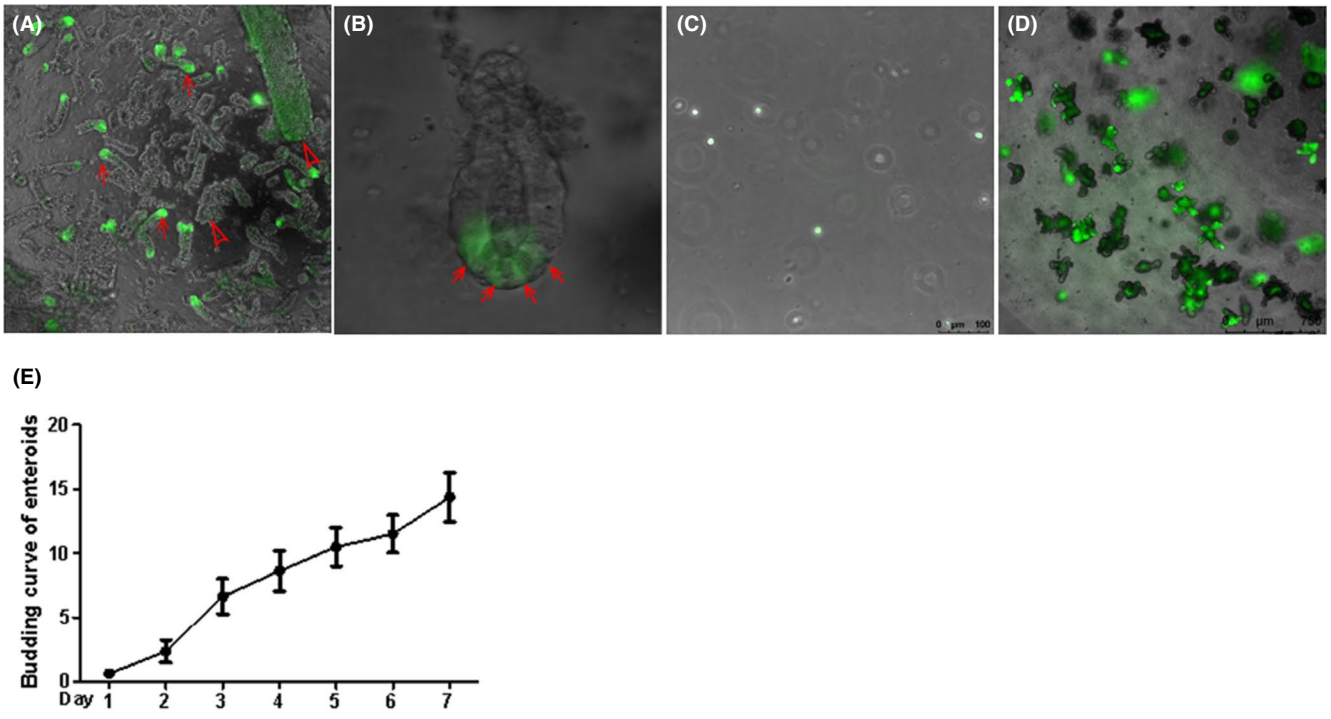

FIGURE 2 Generation of enteroids derived from IESCs over time. A, Intact crypts removed from intestinal basement membrane. Arrows depict crypts while arrowheads indicate portions of villi. B, Intact crypt in high magnification image. Arrows depict stem cells. C, Sorted GFP^{hi} cells from the small intestine of Lgr5 reporter mice. D, Microscopic images of in vitro growth of crypts over 7 d. E, The number of buds was counted daily in a minimum of 10 enteroids per well, result was expressed as a graph of crypt expansion, showing the number of crypt buds vs time

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

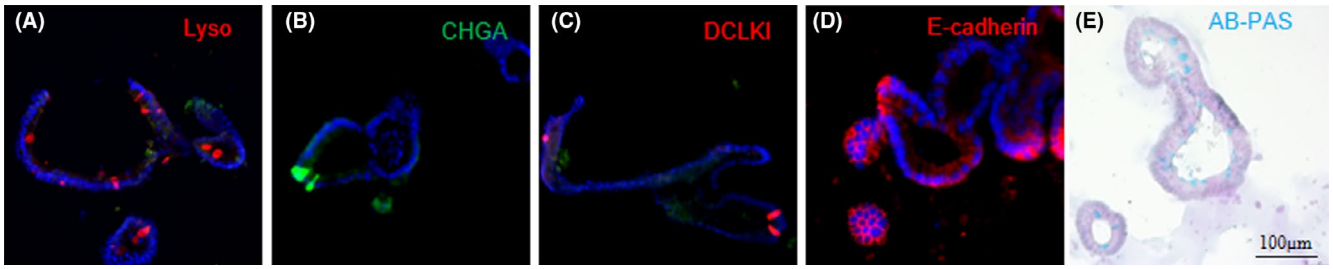

FIGURE 3 IEC lineages from a single Lgr5^{hi}-derived enteroids. Frozen sections were stained by anti-lysozyme (Lyso, Paneth cells), anti-chromogranin A (CHGA, enteroendocrine cells), anti-Dclk1 (Tuft cells), anti-E-cadherin (epithelial cells), and AB-PAS (goblet cells) counterstain, DAPI (blue)

(Minneapolis, MN). 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 Ca^{2+} and Mg^{2+} (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 CO_2 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 CO_2 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 mm^2 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. Jejunum is easy to dissociate after shaking for about 4 minutes; ileum or colon might need 10-15 minutes.
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 × 10^7 cells in a polypropylene tube with 2 mL organoid growth medium.
10. IESCs are isolated as Lgr5 GFP^{hi} with a BD FACS Aria II cell sorter. Lgr5 GFP^{hi}, GFP^{low} 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).[11] The green autofluorescence can be excluded by plotting the GFP channel against the phycoerythrin channel. (Figure 2C).
11. Sorted Lgr5 GFP^{hi} 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).[10]
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% CO_2 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% paraformaldehyde (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 μmol/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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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

XNH, QW, and RXL conceived and designed the study; RXL, HFL and JC carried out experimental work and data analysis; RXL wrote the initial draft of the manuscript. All authors contributed to revising the manuscript and gave final approval for publication.

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