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Quantification of Proliferative and Dead Cells in Enteroids

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TITLE:

Quantification of Proliferative and Dead Cells in Enteroids

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KEYWORDS:

enteroids, intestine, flow cytometry, proliferation, EdU, propidium iodide

SUMMARY:

The presented protocol uses flow cytometry to quantify the number of proliferating and dead cells in cultured mouse enteroids. This method is helpful to evaluate the effects of drug treatment on organoid proliferation and survival.

ABSTRACT:

The intestinal epithelium acts as a barrier that prevents luminal contents, such as pathogenic microbiota and toxins, from entering the rest of the body. Epithelial barrier function requires the integrity of intestinal epithelial cells. While epithelial cell proliferation maintains a

continuous layer of cells that forms a barrier, epithelial damage leads to barrier dysfunction. As a result, luminal contents can across the intestinal barrier via an unrestricted pathway. Dysfunction of intestinal barrier has been associated with many intestinal diseases, such as inflammatory bowel disease. Isolated mouse intestinal crypts can be cultured and maintained as crypt-villus-like structures, which are termed intestinal organoids or “enteroids”. Enteroids are ideal to study the proliferation and cell death of intestinal epithelial cells in vitro. In this protocol, we describe a simple method to quantify the number of proliferative and dead cells in cultured enteroids. 5-ethynyl-2’-deoxyuridine (EdU) and propidium iodide are used to label proliferating and dead cells in enteroids, and the proportion of proliferating and dead cells are then analyzed by flow cytometry. This is a useful tool to test the effects of drug treatment on intestinal epithelial cell proliferation and cell survival.

INTRODUCTION:

A fundamental function of intestinal epithelial cells is to protect the entry of luminal contents such as pathogenic bacteria and toxins[1,2]. To perform such a function, intestinal stem cells continuously proliferate and differentiate into a variety of epithelial cells, including enterocytes and secretory cells, which form a barrier by forming tight connections[3]. The rapid renewal of intestinal epithelial cells requires strict coordination of cell proliferation, cell differentiation, and cell death[4,5]. Reduced cell proliferation or excessive cell death leads to epithelial damage and compromised barrier function[1,6]. Dysfunction of intestinal barrier has been associated with inflammatory bowel diseases[7,8].

A method to culture intestinal crypts has been previously developed. Using this technique, isolated mouse crypts grow into intestinal organoids (enteroids), which have crypt-villus like structures and contain all intestinal epithelial cell lineage[9,10]. 5-Ethynyl-2'-deoxyuridine (EdU) is a thymidine analog that is capable of replacing thymine (T) in DNA that is undergoing replication during cell proliferation. The proliferative cells can be quickly and accurately labeled by EdU staining. Propidium iodide (PI) is an analog of ethidium bromide that releases red fluorescence upon insertion into double-stranded DNA. PI specifically detects dead cells, since it only passes through the damaged cell membrane.

In this protocol, we first describe how to isolate crypts from the murine small intestine then culture them as enteroids in vitro. We then describe how to analyze the proliferative and dead cells in enteroids by EdU and PI incorporation and flow cytometry.

PROTOCOL:

This protocol was approved by the Animal Care and Use Committee of Cambridge-Suda Genomic Resource Center (CAM-SU) at Soochow University.

1. Intestinal organoid isolation and culture

1.1. Isolation of intestinal crypts and enteroid culture

1.1.1. Euthanize an 8-week-old wild-type mouse with CO_2 inhalation. Use tissue forceps and fine iris scissors to dissect out approximately 8 cm of ileum.

1.1.2. Flush out using a syringe with gavage feeding needle with about 40 mL of ice-cold Dulbecco's phosphate-buffered saline (DPBS), then cut lengthwise with scissors and open the ileum.

1.1.3. Cut the ileum into small (0.5−1.0 cm) pieces. Place the pieces in 5 mL of sterile ice-cold DPBS in a 15 mL conical tube, then rock for 5 min on ice.

1.1.4. Use a pipette controller to aspirate the DPBS and replace it with 10 mL of cold buffer 1 (2 mM EDTA in DPBS). Rock for 30 min on ice.

1.1.5. Use the pipette controller to aspirate buffer 1 and replace with 10 mL of cold buffer 2 (54.9 mM D-sorbitol, 43.4 mM sucrose in DPBS). Shake for 2−3 min by hand (~80 shakes/min).

1.1.6. Take a 20 µL droplet of buffer 2 contents after shaking and inspect under a microscope.

1.1.7. Filter buffer 2 contents with a 70 µm sterile cell strainer, then collect the filtered buffer with a 50 mL conical tube.

1.1.8. Pipette 20 µL of filtrate onto the slide to count the crypts. Transfer a sufficient volume of buffer 2 from step 1.1.7 to ensure that there are ~500 crypts/well.

1.1.9. Spin down at 150 x *g* for 10 min at 4 °C. Once spinning is finished, carefully aspirate the supernatant.

1.1.10. Suspend crypts in 50 µL of basement membrane matrix (e.g., Matrigel) per 500 crypts, pipette up and down (being careful to avoid bubbles), then place a 50 µL droplet of basement membrane matrix/crypt mix in the center of one well in a 24 well plate. Incubate for 30 min at 37 °C to polymerize basement membrane matrix.

1.1.11. After 30 min of polymerization, carefully add 600 µL of minigut media (advanced Dulbecco's modified Eagle medium [DMEM]/F12, 2 mM L-alanine-L-glutamine, pen/strep [100 units/mL], 10 mM Hepes, N2 supplement [1:100], B27 supplement [1:50] with epidermal growth factor [EGF; 50 ng/mL], Noggin [100 ng/mL], R-spondin [500 ng/mL], and Y27632 [10 µM]) to each well, then return the plate to the 37 °C incubator. Observe under a microscope each day, and change the media every 2−3 days.

1.2. Enteroid passaging

NOTE: For long cultures, enteroids should be split approximately each week.

1.2.1. To passage the enteroids, place the tissue culture plate on ice. Aspirate the media and

add 1 mL of cold DPBS to each well. Use a P1000 tip to pipette up and down until no solid basement membrane matrix chunks remain.

1.2.2. Pass up and down once through a 1 mL insulin syringe (27 G) and into a 15 mL conical tube. Spin down for 5 min at 150 x *g* at 4 °C.

1.2.3. Use pipette to remove DPBS. Resuspend in 50 µL of basement membrane matrix/well.

1.2.4. Place the plate in a 37 °C incubator for 30 min to allow basement membrane matrix to polymerize. Overlay each well with 600 µL of ENR media (minigut media, 50 ng/mL EGF, 100 ng/mL Noggin, 500 ng/mL R-spondin), then return the plate to the incubator.

2. Flow cytometry analysis of EdU-positive cells in enteroids

NOTE: **Figure 1** shows the workflow for flow cytometry analysis of EdU-positive cells in enteroids.

2.1. Incubation of enteroids with EdU

2.1.1. Grow enteroids from step 1.2.4 for 5–7 days. Passage enteroids into a new 24 well plate in a splitting ratio of 1:2. Add 600 µL of ENR medium to each well. Incubate enteroids for 4–5 days in a 37 °C incubator.

2.1.2. Set the control group (untreated enteroids) and experimental group (enteroids treated with 5 ng/mL interleukin 22 [IL-22]). Prepare at least three replicates for each group.

2.1.3. Add EdU to the ENR medium to prepare EdU medium with a concentration of 50 µM. Add 600 µL of EdU medium to each well and incubate for 2 h in a 37 °C incubator. Set one well of enteroids without EdU treatment as a negative control for background subtraction.

2.2. Harvesting of enteroids from basement membrane matrix

2.2.1. Use a pipette controller to aspirate EdU medium and wash 1x with DPBS. Add 1 mL of DPBS.

2.2.2. Use P1000 pipette tip and pipette up and down until no solid basement membrane matrix chunks remain. Transfer to a 15 mL tube and spin down at 300 x *g* for 5 min. Discard the supernatant.

2.2.3. Add 500 µL of cell-dissociation enzymes (**Table of Materials**) and incubate for 15 min at 37 °C. Use P200 pipette tip and pipette up and down to break enteroids into single cells.

2.2.4. Add 3 mL of DMEM medium containing 10% fetal bovine serum (FBS) and repeatedly pipette with a P1000 pipette tip.

2.2.5. Spin down at 300 x *g* for 5 min and aspirate the supernatant medium. Resuspend the cells with 1 mL of DPBS.

2.3. Fixation and permeabilization of cells

2.3.1. Transfer the cell suspension to a 1.5 mL tube, spin down at 300 x *g* for 5 min, and discard the supernatant.

2.3.2. Resuspend cells with 1 mL of 4% paraformaldehyde (PFA), fix for 15 min at room temperature (RT), and spin down at 300 x *g* for 5 min. Aspirate the supernatant with a pipette tip, wash 1x with DPBS, and spin down to remove the supernatant.

2.3.3. Resuspend cells with 1 mL of 0.5% nonionic surfactant. Incubate for 10 min at RT.

2.3.4. Spin down at 300 x *g* for 5 min and aspirate the supernatant. Wash 1x with DPBS and spin down to remove the supernatant.

2.4. Detection of EdU

2.4.1. Prepare stock solutions for each component in advance: 1) working solution of the Alexa Fluor azide, 2) working solution of 1x EdU reaction buffer, and 3) 10x stock solution of EdU buffer additive.

2.4.2. Prepare 1x EdU buffer additive by diluting the 10x solution 1:10 in deionized water. Prepare this solution fresh.

2.4.3. Prepare reaction cocktail according to **Table 1**.

NOTE: Add the ingredients in the order listed in the table. Use the reaction cocktail within 15 min of preparation.

2.4.4. Add 100 µL of reaction cocktails to each 1.5 mL tube. Resuspend the cells, protect from light, incubate for 30 min at RT.

2.4.5. Centrifuge at 300 x *g* for 5 min and aspirate the reaction solution with pipette tip gently.

2.4.6. Add 0.5% nonionic surfactant penetrant to each tube to wash 1x at RT. Centrifuge at 300 x *g* for 5 min, aspirate the supernatant with pipette tip gently, and resuspend the cells in 1 mL of DPBS.

2.5. Analysis of cells by flow cytometry

2.5.1. Filter the resuspended cells with a 40 µm strainer, then collect the filtered cells with a

15 mL conical tube. Perform FACS on-machine detection as soon as possible.

NOTE: If conditions are limited, the cell stained samples should be stored in the dark at 4 °C and flow-tested within 3 days.

2.5.2. Select the appropriate channel (according to the type of Alexa Fluor azide in the EdU kit; here, red channel) and voltage (here, ~150–350 V) for flow cytometry analysis.

2.5.3. Gating strategy

2.5.3.1. Draw an FSC-A (x-axis) *vs.* SSC-A (y-axis) pseudocolor plot and distribute most of the cells to the visible range of the dot map by adjusting the voltage. Select the cell population (R1) and exclude the cell debris in the bottom left corner.

2.5.3.2. From the R1 cell population, establish an FSC-A (x-axis) *vs*. FSC-H (y-axis) pseudocolor plot, and set the gate to select single cells (R2) to exclude cell clumps.

2.5.3.3. From the R2 cell population, establish the fluorescence intensity (x-axis) *vs*. cell number (y-axis) plot, and use the negative control to set the gate. The region of the fluorescent signal is a positive cell region (R3). Compare the ratio of EdU-positive cells (R3) between the experimental and control groups.

3. Flow cytometry analysis of PI-positive cells in enteroids

NOTE: **Figure 2** shows the workflow for flow cytometry analysis of PI-positive cells in enteroids.

3.1. Incubation of enteroid cells with PI

3.1.1. Grow enteroids from step 1.2.4 for 5–7 days. Passage enteroids into a new 24 well plate in a splitting ratio of 1:2. Add 600 µL of ENR medium to each well. Incubate enteroids for 4−5 days in a 37 °C incubator.

3.1.2. Set the control group (untreated) and experimental group (IL-22 treated), using at least three replicates for each group.

3.1.3. Add PI to the ENR medium to prepare PI medium with a concentration of 3 µM.

3.1.4. Add 600 µL of PI medium to each well and incubate for 30 min in a 37 °C incubator. Set one well of enteroids without PI treatment as a negative control for background subtraction.

3.2. Harvest enteroids from basement membrane matrix following steps 2.2.1−2.2.5.

3.3. Analysis of cells by flow cytometry

3.3.1. Filter the resuspended cells with a 40 µm strainer and collect the filtered cells with a 15 mL conical tube.

3.3.2. Perform FACS on-machine detection as soon as possible.

NOTE: If conditions are limited, the cell stained samples should be stored in the dark at 4 °C and flow-tested within 3 days.

3.3.3. Select the appropriate channel (here, red channel) and voltage (here, ~150–350 V) for flow cytometry analysis.

3.3.4. Use the same gating strategy as described in section 2.5.3.

REPRESENTATIVE RESULTS:

Small intestinal crypts were isolated and cultured as enteroids in basement membrane matrix. Enteroids started to form buds 2 days after isolation. On day 6, enteroids had many buds with lots of debris (dead cells) in the lumen. Enteroids were ready to be passaged at this stage (**Figure 3**).

Numerous studies have shown that inflammatory cytokines are essential for the maintenance of intestinal epithelial homeostasis. Abnormal expression of inflammatory cytokines is closely associated with the occurrence of inflammatory bowel diseases[11]. For instance, our previous study showed that IL-22 promotes proliferation of transit-amplifying cells but also depletes $Lgr5^+$ stem cells[12].

Enteroids were treated with IL-22 for 3 days, after which the synthetic DNA was labeled with EdU to indicate cell proliferation. IL-22-treated enteroids displayed an increased number of EdU^+ cells (**Figure 4A**). IL-22 increased proliferating cells from 40.1% to 83.5% as analyzed by flow cytometry (**Figure 4B**). IL-22 treatment also increased the cell death in enteroids, indicated by PI staining (**Figure 5A**). IL-22 increased dead cells from 4.9% to 16.2% as analyzed by flow cytometry (**Figure 5B**).

FIGURE LEGENDS:

Figure 1: Workflow diagram for flow cytometry analysis of EdU-positive cells in enteroids.

Figure 2: Workflow diagram for flow cytometry analysis of PI-positive cells in enteroids.

Figure 3: Brightfield images of enteroids at 2, 4, and 6 days post-crypt isolation. Scale bar = 100 µm.

Figure 4: IL-22 increases enteroids proliferation. (**A**) Enteroids were cultured in medium without or with IL-22 (5 ng/mL) for 3 days and incubated with EdU (red) for 1 h. Scale bar = 100 µm. (**B**) Flow cytometry data from enteroids cultured without (left) or with (right) IL-22.

Data are representative of three separate experiments.

Figure 5: IL-22 promotes cell death in enteroids. (**A**) Enteroids were cultured in medium without or with IL-22 (5 ng/mL) for 3 days and stained with propidium iodide (red). Scale bar = 100 µm. (**B**) Flow cytometry data from enteroids cultured without (left) or with (right) IL-22. Data are representative of three separate experiments.

Table 1: EdU reaction cocktail.

DISCUSSION:

This protocol details the steps necessary for the culture of enteroids in vitro and quantification of EdU- and PI-positive cells in the enteroids by flow cytometry. There are several advantages of this strategy. First, EdU labelling is used to detect proliferating cells in enteroids. Compared with traditional BrdU assay, EdU labelling method is faster, more sensitive, and more accurate. EdU is very similar to thymine (T), which replaces thymine in DNA synthesis during cell division. Compared to the BrdU antibody, EdU is easier to diffuse into the cell, and the detection of EdU does not require DNA denaturation and an antigen-antibody reaction. Secondly, flow cytometry analysis can quickly and accurately quantify the proliferating (EdU^+) and dead (PI^+) cells in enteroids.

To successfully perform the entire procedure, there are critical aspects to be considered. First, it is important to culture enteroids under sufficient conditions. Well-grown enteroids should have plenty of buds, which contain proliferative cells. Second, it is important to split enteroids in a timely manner. Debris accumulated in the lumen contains dead cells, which can be stained with PI. This is detrimental for the following flow cytometry analysis. Third, due to the multiple steps (i.e., cell staining, cell fixation, membrane rupture, and centrifugation), cells can be easily lost during the procedure. Thus, it is important to collect a sufficient number of enteroids. It is important to combine 2−3 wells (in a 24 well plate) of enteroids before fixation. Lastly, it is critical to add ingredients to the buffer in order to detect EdU, otherwise the reaction will not proceed optimally.

In summary, the protocol details steps for the culturing of mouse enteroids in vitro and the quantification of proliferating and dead cells by flow cytometry. Enteroids are useful tools for disease modelling and therapeutic drug discovery. This protocol helps exploration of the effects of inflammatory cytokines, pathogen, and drugs on cell proliferation and cell survival in enteroid culture models.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

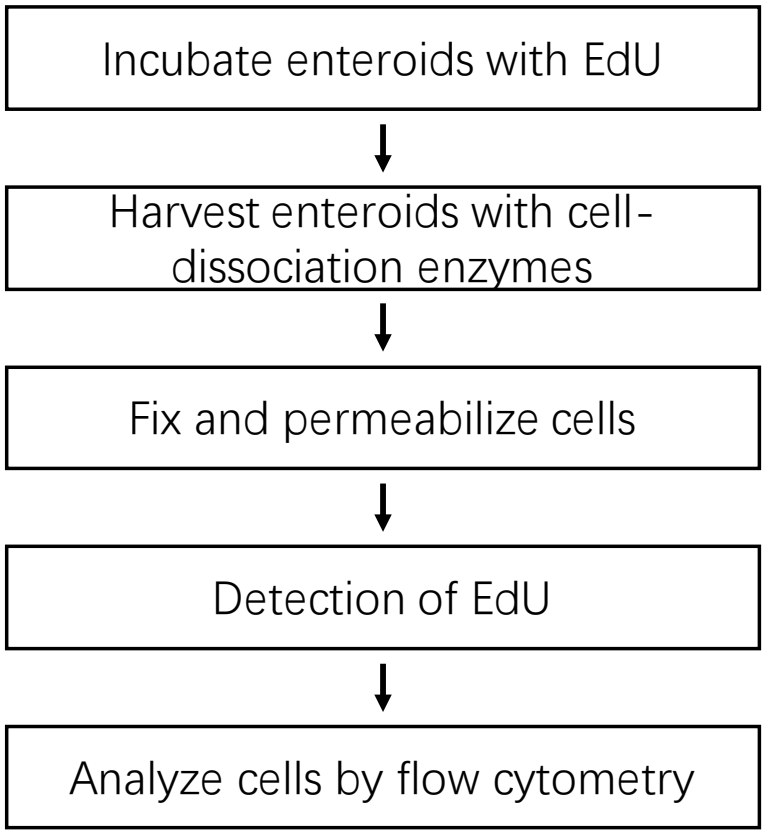


Figure 2

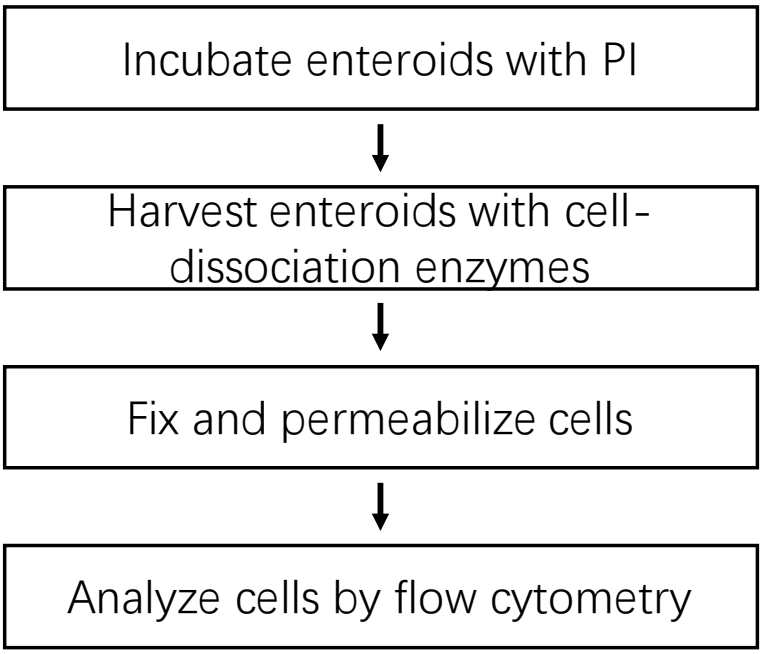


Figure 3

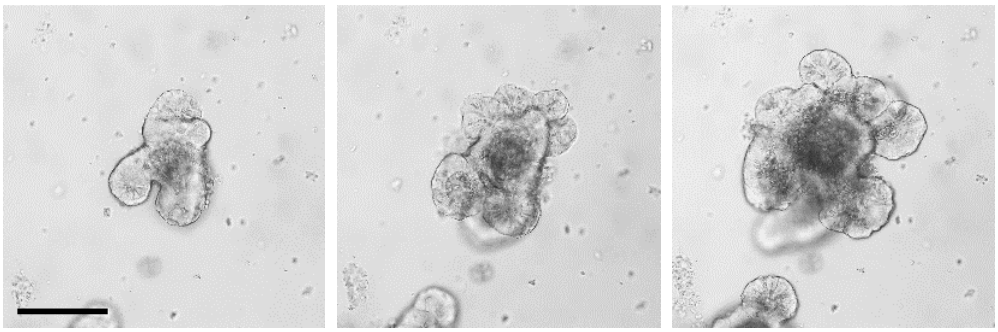


Figure 4

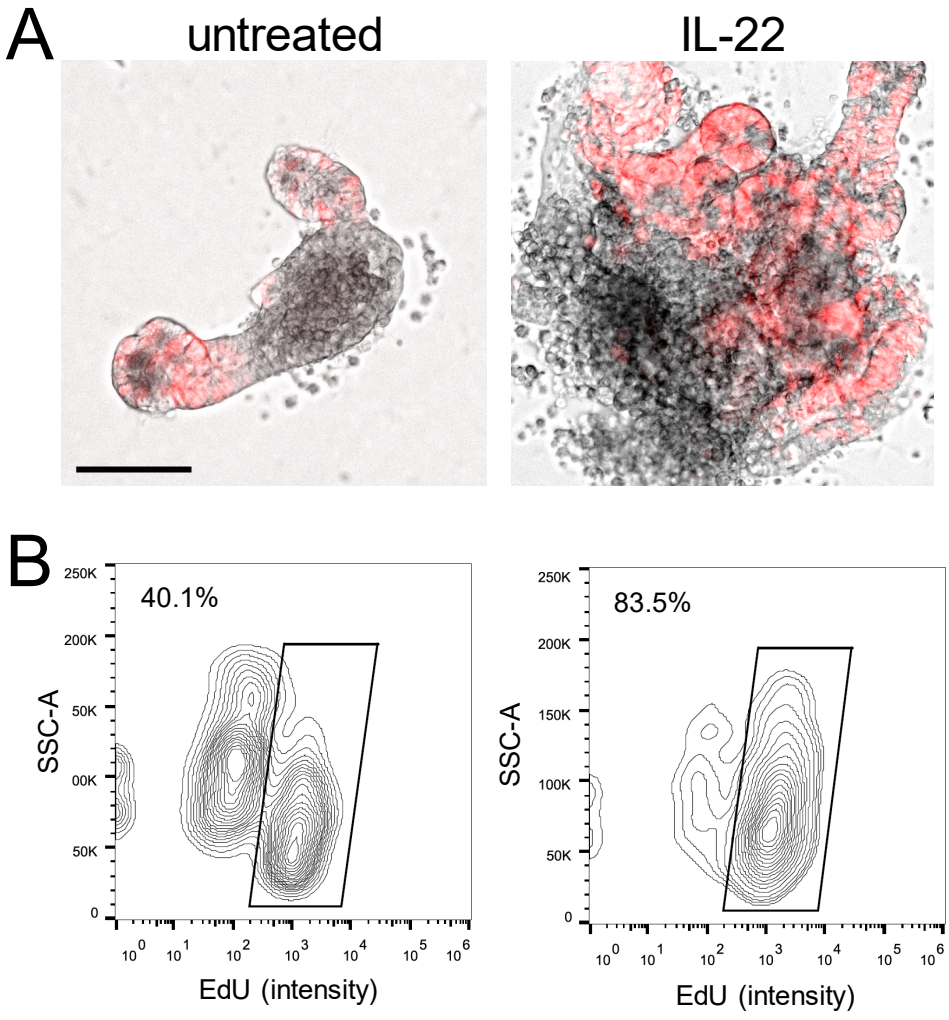
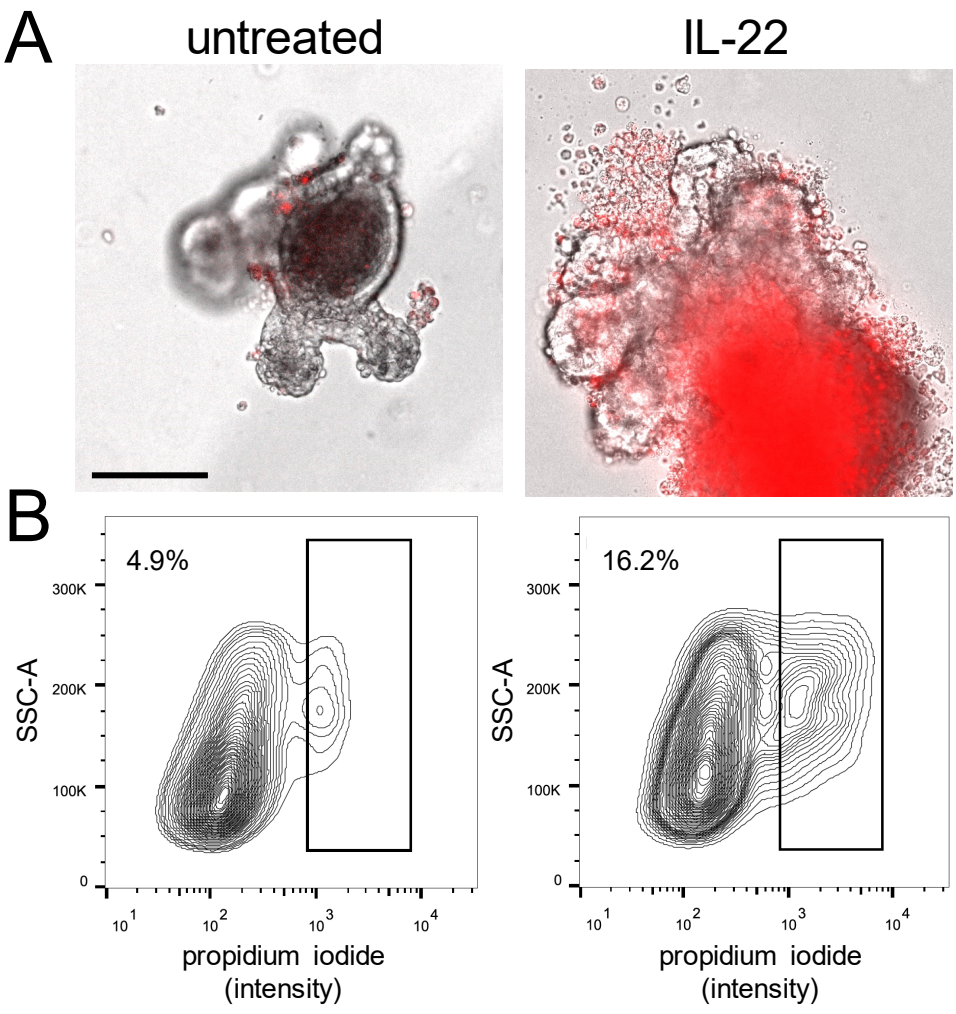


Figure 5



Table

Reaction components	Number of wells of 24-well plates					
	1	2	5	10	20	50
1x Reaction buffer	86 μL	172 μL	430 μL	860 μL	1.72 mL	4.3 mL
$CuSO_4$	4 μL	8 μL	20 μL	40 μL	80 μL	200 mL
Alexa Fluor azide	0.25 μL	0.5 μL	1.25 μL	2.5 μL	5 μL	12.5 mL
Reaction buffer additive	10 μL	20 μL	50 μL	100 μL	200 μL	500 μL
Total volume	100 μL	200 μL	500 μL	1 mL	2 mL	5 mL
Note: Add the ingredients in the order listed in the table.						

Name of Material/Equipment

15 ml centrifuge tube
22 G gavage needle
24-well plate
40 mm sterile cell strainer
50 ml centrifuge tube
70 mm sterile cell strainer
Advanced DMEM/F-12
Attune NxT Acoustic Focusing Cytometer
B-27 Supplement
Buffer 1

Buffer 2

C57/B6 mice
Cell-dissociation enzymes (TrypLE)
Centrifuge
Centrifuge
Centrifuge
Click-iT Plus EdU Alexa Fluor 594 Imaging Kit
CO_2 incubator
DPBS
D-sorbitol
EDTA

ENR media

Fetal Bovine Serum (FBS)
Fine Iris Scissors
Fluorescence microscope
GlutaMAX Supplement
Goat Serum
HDMEM
HEPES
Matrigel

Minigut media

N2 supplement
Nonionic surfactant (Triton X)
Operating Scissor (12.5 cm)
Paraformaldehyde (PFA）

Penn/Strep
Phase contrast microscope
Propidium iodide
Recombinant EGF
Recombinant Mouse Noggin
Recombinant Mouse R-Spondin 1
Recombinant Murine IL-22
Sucrose
Tissue Forceps
Y-27632 2HC1

Company	Catalog Number
Corning	430791
VWR	20068-608
Nunc	142475
BD	352340
Corning	430829
BD	352350
GIBCO	12634010
Invitrogen	A24863
GIBCO	17504044

Nanjing Biomedical Research Institute of Nanjing University	
Life technologies	12605-010
Eppendorf	5424
Eppendorf	5424R
Eppendorf	5810R
Life technologies	C10639
Panasonic	MCO-18AC
GIBCO	14190144
BBI	SB0491
BBI	EB0185

Gibco	10270-106
Tansoole	2037454
Olympus	FV1000
GIBCO	35050-061
Life technologies	16210-064
Hyclone	SH30243.01B
Sigma	H4034
Corning	356231

R&D	AR009
BBI	TB0198-500ML
Tansoole	2025785
sigma	158127-500g

Invitrogen	15140-148
Nikon	TS1000
Sigma	P4170-25MG
PeproTech	315-09
PeproTech	250-38
R&D	3474-RS-050
PeproTech	210-22-10
BBI	SB0498
Tansoole	2026704
Selleck	S1049

Comments/Description

2 mM EDTA in DPBS

54.9 mM D-sorbitol, 43.4 mM sucrose in DPBS

Minigut media, 50 ng/ml EGF, 100 ng/ml Noggin, 500 ng/ml R-spondin

Advanced DMEM/F12, 2 mM Glutamax, Penn/Strep (100 units/ml), 10 mM Hepes, N2 supplement (1:100), B27 supplement (1:50)

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Ref: JoVE60501
Title: Quantification of proliferating and dead cells in enteroids

Dear Dr. Cao,

We greatly appreciate the comments and constructive issues raised by the editor and reviewers. We have diligently responded with text editing to address the raised issues as described in the marked manuscript and detailed responses to reviewers below. These changes have improved the manuscript so we express our gratitude to the reviewers for their careful reviews.

Kind regards,
Weiqi He
Soochow University

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response: We have proofread the manuscript thoroughly.

2. Authors and affiliations: Please provide an email address for each author in the manuscript.
Response: We have added the email address for each author in the title page.

3. Summary/Short Abstract: Please expand to include a general description of the method and its applications.
Response: We have modified the description in Short Abstract.

4. Please define acronyms/abbreviations upon first use in the main text.
Response: Done.

5. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.
Response: We have added this information in line 90-91.

6. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
Response: This has been amended.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
Response: Done.

8. Please specify all surgical tools used throughout the protocol.
Response: We specify all surgical tools in line 94.

9. Please provide all volumes and concentrations used throughout.
Response: We have added this information in the manuscript.

10. For culture media and buffer (e.g., Buffer 1 and 2), please spell out at first use and provide composition. If they are purchased, please cite the Table of Materials.
Response: The composition of culture media and buffer are added in the text (line 100-103, 116-118, 130-131). This information is also provided in the supplementary material.

11. 1.1.1: Please specify the source of ileum.
Response: The ileum is isolated from 8-week-old wild-type mice (line 95).

12. 1.1.2: Please describe how this is done. What volume of DPBS is used?
Response: We have described this in the text. Approximately 40 mL buffer1 was used.

13. 1.1.5: What volume of buffer 1 is used?
Response: 10 mL buffer1 was used.

14. 2.1.2: Please specify what the control group and the experimental group are.
Response: We have specified the control group and the experimental group (line 138-139).

15. 2.3.2: At what temperature are the cells fixed?
Response: The cells are fixed at room temperature.

16. 3.3.2: Please describe gating strategies.
Response: We have added the gating strategies in the revised manuscript as the step 2.5.4.

17. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Response: Done.

18. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal

of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
Response: Done.

19. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
Response: Done.

20. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Response: Done.

21. Please replace commercial language (TrypLE, TritonX-100) with generic terms.
Response: Done.

22. References: Please do not abbreviate journal titles; use full journal name.
Response: Done.

23. Figure 1: Please replace “TrypLE” with a generic term.
Response: Done.

24. Figure 4 and Figure 5: Please define scale bars in the figure legend.
Response: We have defined scale bars in figure legends.

25. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
Response: The table has been removed and uploaded separately.

26. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.
Response: We have uploaded each figure individually as .ai file.

27. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.
Response: This has been confirmed.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
In the manuscript "Quantification of proliferating and dead cells in enteroids", Authors clearly descripted the protocol of Intestinal organoids isolation, culture and passage, how to analysis of EdU positive cells in enteroids by Flow cytometry, and those information are very useful and important for analysis human Intestinal disease in clinic, such as analysis the effects of inflammatory cytokines,
pathogen, and drugs on Intestinal disease.

Minor Concerns:
1. Authors should describe a little more significance of this protocol on the clinical analysis in introduction or discussion part.
Response: Thanks for your careful review and valuable suggestion. We have described some more significance of this protocol on the clinical analysis in the discussion part.

Reviewer #2:

Manuscript Summary:
In this manuscript, Li et al describe their methods to analyze cell viability (i.e. proliferation by EdU labeling) and cell death (i.e. via propidium iodide incorporation) in murine enteroids. Overall changes in either proliferation or cell death were quantified by flow cytometry. These methods are used to compare the effects of IL-22, a cytokine known to increase proliferation of transit amplifying cells, on cell proliferation and cell death in mouse ileal enteroids.

Major Concerns:
While methods designed to profile cell viability/cell death in primary ex vivo intestinal epithelial cultures are useful for comparing a variety of pathological challenges to the gut barrier, the methods detailed in this manuscript are basic protocols that have been used extensively in many other publications. In addition, there are several areas that lack sufficient detail in regards to reproducibility and uniform results, further explanations as to why methods described in this manuscript improve and/or differ upon already published methods, and validation of results obtained by these methods.
Response: Thanks for pointing this out. We believe that the methods described in our manuscript are basic protocols that have been universally used in many laboratories. Introduction of gold standard protocols is one of scopes of the video journal. In this manuscript, we aim to provide a basic protocol for quantifying proliferating and dead cells in cultured enteroids. We have improved the description of the methods step by step to ensure the reproducibility of the protocol.

Minor Concerns:

1. IL-22 treatment both increases Edu and PI labeling. Based on their previous publication, and those of others describing the same effect, the IL-22 effects should be due to increased proliferation of transit amplifying cells and a loss of Lgr5+ dividing cells. Based on their flow results, are the cells labeled with PI specifically Lgr5+ cells and are the EdU cells specifically TA cells?
Response: IL-22 increases proliferation of TA cells and depletes Lgr5+ stem cells. However, we believe that: Besides TA cells, Lgr5+ cells, which are highly proliferative, should be also labeled with EdU. It seems that IL-22 also increases the turnover of epithelial cell and shedding of dead cells. Therefore, PI labels dying Lgr5+ stem cells and shedding cells of enteroids with IL-22 treatment.

2. It is well established in murine enteroids that "dark" lumens, which include dead epithelial cells shed into the lumen by anoikis, are a typical indicator of enteroids that require passage; otherwise, they will rupture. In these experiments, Il-22 is exposed for another 3 days. Is the increased PI labeling due to rupture of enteroids or IL-22?
Response: As mentioned above, IL-22 likely increases the turnover of epithelial cell and shedding of dead cells into the lumen. We think that IL-22 leads to enteroids rupture by increasing cell debris into the lumen and increases PI labeling.

3. Could the authors please have the manuscript reviewed for grammatical errors throughout?
Response: The grammatical errors have been corrected. Thanks.