Video Article

Quantification of Proliferative and Dead Cells in Enteroids

Hua-Shan Li*[1], Shao-Fang Xu*[1], Jian-Ying Sheng*[1], Zhi-Hui Jiang[1], Jing Wang[1], Ning Ding[1], Tao Wang[1], Matthew A. Odenwald[2], Jerrold R. Turner[2,3], Wei-Qi He[1], Hong Xu[1], Juan-Min Zha[1]

[1]Jiangsu Key Laboratory of Neuropsychiatric Diseases and Cambridge-Suda (CAM-SU) Genomic Resource Center, Medical College of Soochow University, Department of Oncology, The First Affiliated Hospital of Soochow University

[2]Department of Pathology, University of Chicago

[3]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School

*These authors contributed equally

Correspondence to: Wei-Qi He at whe@suda.edu.cn, Hong Xu at 13301549066@163.com, Juan-Min Zha at zhajuanmin@suda.edu.cn

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/60501/

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. Isolation of intestinal crypts and enteroid culture

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.
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.
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.
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.
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).
6. Take a 20 µL droplet of buffer 2 contents after shaking and inspect under a microscope.
7. Filter buffer 2 contents with a 70 µm sterile cell strainer, then collect the filtered buffer with a 50 mL conical tube.
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.
9. Spin down at 150 x *g* for 10 min at 4 °C. Once spinning is finished, carefully aspirate the supernatant.
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.
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.

2. Enteroid passaging
   NOTE: For long cultures, enteroids should be split approximately each week.
   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.
   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.
   3. Use pipette to remove DPBS. Resuspend in 50 µL of basement membrane matrix/well.
   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.

1. Incubation of enteroids with EdU
   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. 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.
   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. Harvesting of enteroids from basement membrane matrix
   1. Use a pipette controller to aspirate EdU medium and wash 1x with DPBS. Add 1 mL of DPBS.
   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.
   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.
   4. Add 3 mL of DMEM medium containing 10% fetal bovine serum (FBS) and repeatedly pipette with a P1000 pipette tip.
   5. Spin down at 300 x *g* for 5 min and aspirate the supernatant medium. Resuspend the cells with 1 mL of DPBS.

3. Fixation and permeabilization of cells
   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. 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.
   3. Resuspend cells with 1 mL of 0.5% nonionic surfactant. Incubate for 10 min at RT.
   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.

4. Detection of EdU
   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. Prepare 1x EdU buffer additive by diluting the 10x solution 1:10 in deionized water. Prepare this solution fresh.
   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.
   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.
   5. Centrifuge at 300 x *g* for 5 min and aspirate the reaction solution with pipette tip gently.

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.

5. Analysis of cells by flow cytometry
    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. 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.
    3. Gating strategy
        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. 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.
        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.

1. Incubation of enteroid cells with PI
    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. Set the control group (untreated) and experimental group (IL-22 treated), using at least three replicates for each group.
    3. Add PI to the ENR medium to prepare PI medium with a concentration of 3 µM.
    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.

2. Harvest enteroids from basement membrane matrix following steps 2.2.1−2.2.5.
3. Analysis of cells by flow cytometry
    1. Filter the resuspended cells with a 40 µm strainer and collect the filtered cells with a 15 mL conical tube.
    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. Select the appropriate channel (here, red channel) and voltage (here, ~150–350 V) for flow cytometry analysis.
    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**).

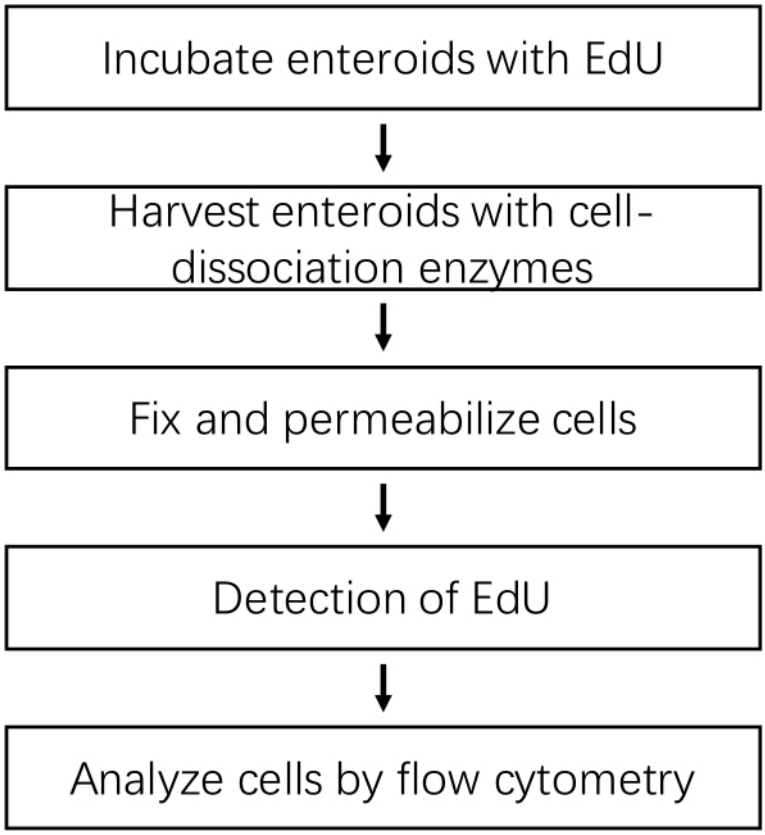

Figure 1: Workflow diagram for flow cytometry analysis of EdU-positive cells in enteroids. Please click here to view a larger version of this figure.

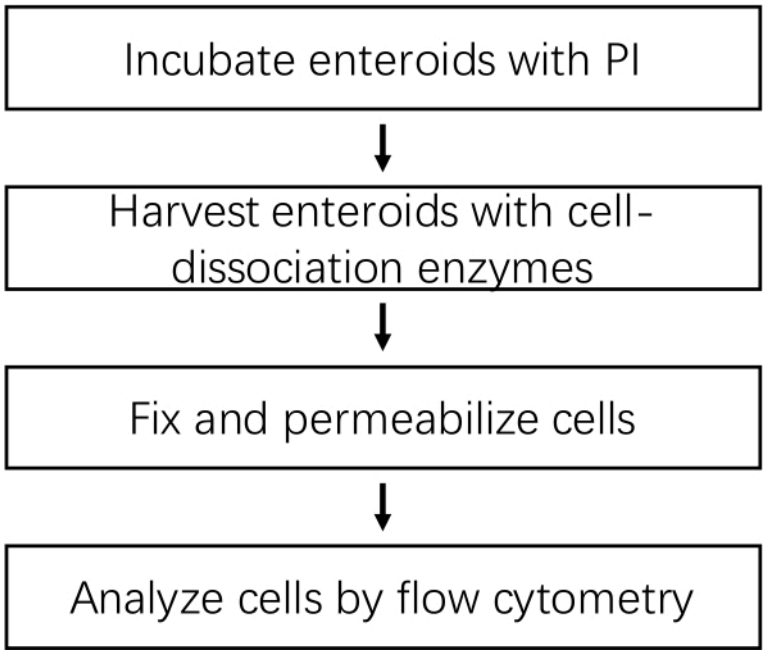

Figure 2: Workflow diagram for flow cytometry analysis of PI-positive cells in enteroids. Please click here to view a larger version of this figure.

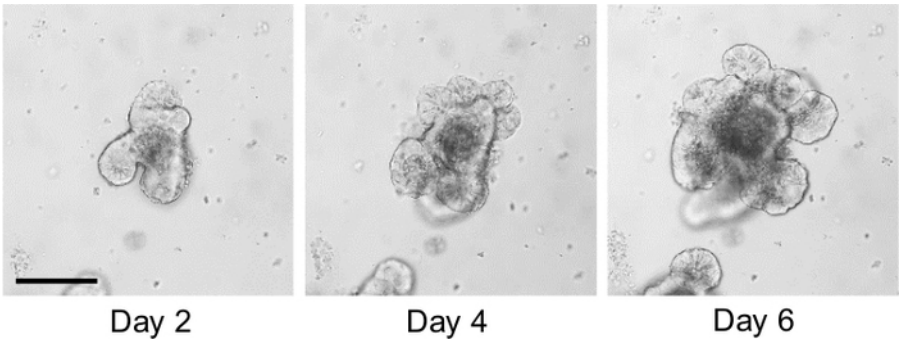

Figure 3: Brightfield images of enteroids at 2, 4, and 6 days post-crypt isolation. Scale bar = 100 µm. Please click here to view a larger version of this figure.

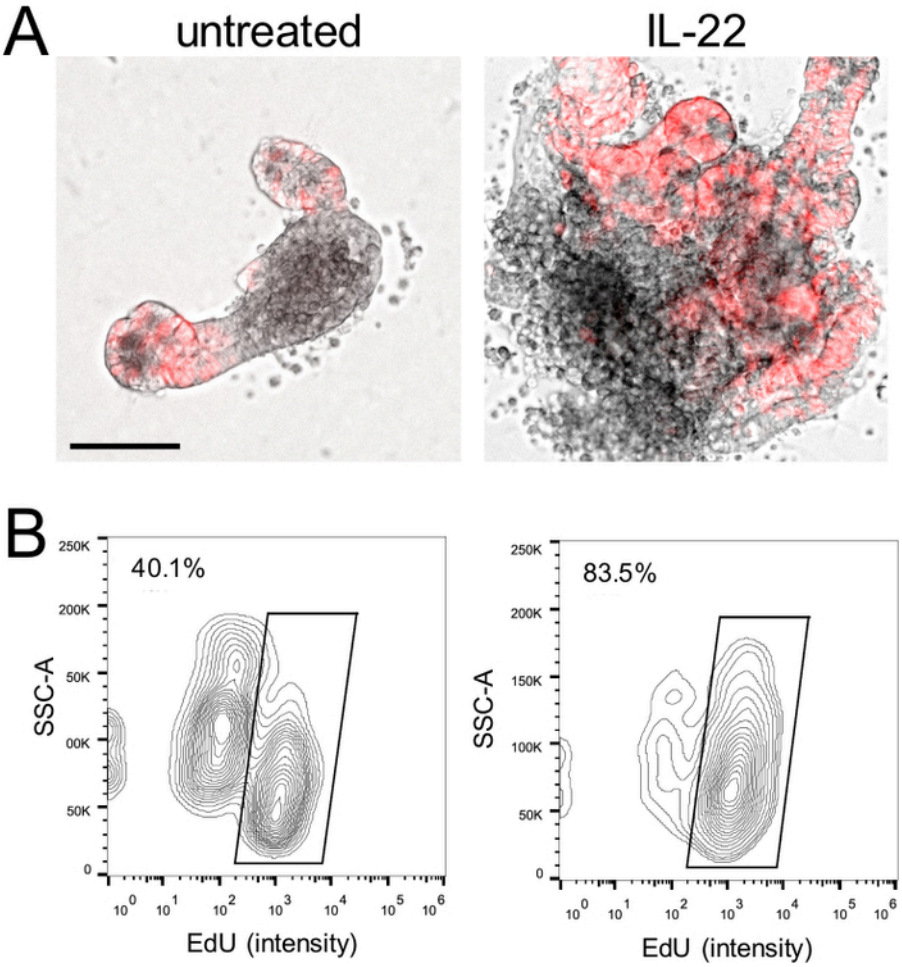

Figure 4: IL-22 increases enteroids proliferation. (**A**) Enteroids were cultured in medium without or with IL-22 (5 ng/mL) for 3 days and incubated with EdU (red) for 1 h. Scale bar = 100 µm. (**B**) Flow cytometry data from enteroids cultured without (left) or with (right) IL-22. Data are representative of three separate experiments. Please click here to view a larger version of this figure.

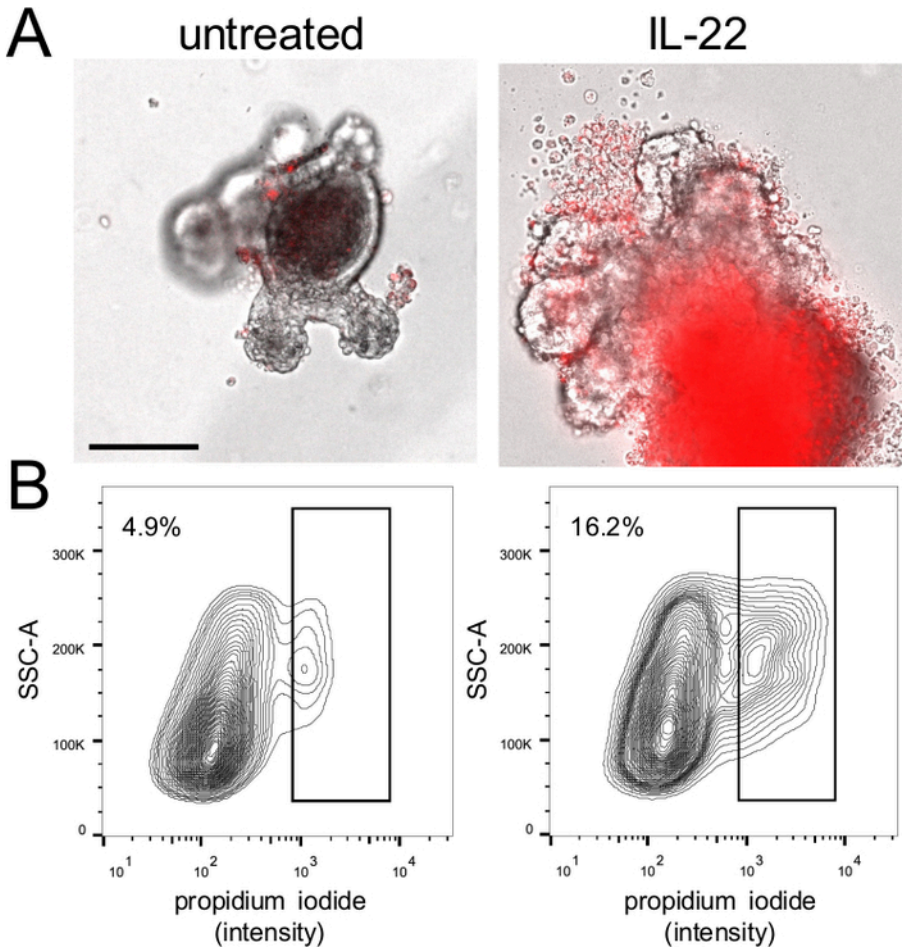

Figure 5: IL-22 promotes cell death in enteroids. (**A**) Enteroids were cultured in medium without or with IL-22 (5 ng/mL) for 3 days and stained with propidium iodide (red). Scale bar = 100 µm. (**B**) Flow cytometry data from enteroids cultured without (left) or with (right) IL-22. Data are representative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

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

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.

Disclosures

The authors have nothing to disclose.

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