Chapter 7

Analysis of Cell Death Induction in Intestinal Organoids In Vitro

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Abstract

The intestinal epithelium has an important function in the absorption of nutrients contained in the food. Furthermore, it also has an important barrier function, preventing luminal pathogens from entering the bloodstream. This single cell layer epithelium is quite sensitive to various cell death-promoting triggers, including drugs, irradiation, and TNF family members, leading to loss of barrier integrity, epithelial erosion, inflammation, malabsorption, and diarrhea. In order to assess the intestinal epithelium-damaging potential of treatments and substances specific test systems are required. As intestinal tumor cell lines are a poor substitute for primary intestinal epithelial cells, and in vivo experiments in mice are costly and often unethical, the use of intestinal organoids cultured from intestinal crypts provide an ideal tool to study cell death induction and mechanisms in primary intestinal epithelial cells. This protocol describes the isolation and culture of intestinal organoids from murine small intestinal crypts, and the quantitative assessment of cell death induction in these organoids.

Key words Apoptosis, Intestinal epithelial cells, Crypts, TNFα, Chemotherapy, MTT, Irradiation, Organoids, Enteroids

1 Introduction

The intestinal epithelium is a tissue with impressive dimensions and important physiological functions. The surface of the human small and large intestinal epithelium comprises several hundred square meters, representing the largest epithelial surface in the human body. The intestinal epithelium has an important function in the digestion and absorption of nutrients. While gastric and pancreatic enzymes contribute the major food digestive enzymatic machinery, the intestinal epithelium also secretes digestive enzymes, which further break down nutrients into absorbable monosaccharides, amino acids, and fatty acids. These and other food components, such as salts, vitamins, but also drugs, are then efficiently absorbed by epithelial cells and transported to the circulation for distribution in the body. Next to absorptive activities the intestinal

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epithelium has also important protective functions. The intestinal lumen is densely populated with an enormous number of harmless commensal bacteria, but also potentially pathogenic microbes and viruses. The intestinal epithelium, together with the mucus layer produced by the goblet cells, provides an important barrier for preventing pathogens from entering the bloodstream. Epithelial cells also transport secretory IgA to the luminal surface, which helps to protect the host from bacterial infections. Furthermore, Paneth cells at the bottom of the small intestinal crypts are a rich source of antibacterial proteins, such as lysozyme and defensins (reviewed in [1]). Last but not least, the intestinal epithelial layer not only contains diverse immature and mature epithelial cells, but is home of a large number of so-called intraepithelial lymphocytes, which contribute to immune regulation and host defense [2].

While this single cell layer epithelium is well adapted for efficient food uptake, it is also very vulnerable to damage, potentially leading to the breakdown of the epithelial barrier function, access of pathogens to circulation and other host tissues, and induction of harmful inflammatory processes and/or bacterial sepsis. Thus, gaps in the epithelial layer due to damage or loss of cells have to be immediately filled by new cells deriving from the fast diving intestinal stem and early progenitor cells [3, 4]. This fast diving cell population in the intestinal crypts is critical for the constant self-renewing of the intestinal epithelial layer. Due to their rapid proliferation and frequent DNA synthesis they are also prone to acquire DNA damage and mutations in critical genes, which may result in the development of cancer, such as colorectal tumors. This rapid proliferation rate also makes them highly susceptible to irradiation and chemotherapeutic drugs that target DNA [5].

The intestinal epithelium is one of the tissues with the highest proliferation rate, and also is a site where extensive physiological and pathophysiological cell death is ongoing. Since the epithelial layer is continuously exposed to damaging stimuli, there is a constant production of new epithelial cells, which are pushed from the bottom of the crypts to the top of the villus in the small intestine, and to the epithelial surface in the large intestine, where they are shed into the lumen. Thus, the life span of an intestinal epithelial cell is only a few days. Detachment of mature epithelial cells from the basal membrane leads to death by anoikis, a detachment-induced form of apoptosis (reviewed in [3]). Even though a very large number of intestinal epithelial cells die by anoikis every moment, only few dying cells are observed within the epithelial layer under physiological conditions [6]. Likely, the rapid shedding of dying cells into the lumen, and their replacement by new cells from the crypts ensures maintenance of the epithelial barrier function.

A completely different situation is seen under pathophysiological conditions. The high sensitivity of intestinal epithelial cells

to a variety of drugs, treatments, and biological apoptosis inducers results in massive cell death induction in mature and immature epithelial cells in a variety of pathophysiological conditions [6, 7]. As mentioned above, intestinal crypt cells are amongst the fastest dividing cells in the human body, and the associated fast DNA replication makes them prime targets of chemotherapeutic drugs or irradiation [5, 8]. Thus, not surprisingly damage of the intestinal epithelium is one of the most common side effects of anticancer treatments by chemotherapy or whole body irradiation. Similarly, the intestinal epithelium is also highly susceptible to a variety of immune cell effector molecules. For example, activation of T cells and macrophages leads to the expression of members of the TNF family, such as TNFα itself and Fas ligand (FasL), which have potent apoptosis-inducing activities in intestinal epithelial cells [7–9]. As a consequence, immune cells activation in mice in vivo upon injection of T cell-activating anti-CD3ε antibody or macrophage-activating lipopolysaccharide (LPS) results in FasL and TNFα expression, massive intestinal crypt cell death and mature epithelial cell shedding, and a breakdown of the epithelial barrier function. Immune cell-mediated intestinal epithelium damage is also frequently observed in the course of immunopathological disorders, such as intestinal graft-versus-host disease, inflammatory bowel disease, bacterial and viral infections, and sepsis [10, 11].

As the excessive permeabilization of the intestinal epithelium due to uncontrolled and extensive cell death induction may have devastating consequences, including death, a better understanding of the signaling processes involved in cell death induction in intestinal crypt and mature epithelial cells is a prerequisite for the development of novel strategies and treatments aiming at preventing such pathological processes. Unfortunately, thus far good model systems have been lacking. As mentioned above, upon isolation, primary intestinal epithelial cells rapidly die by apoptosis, making it difficult to study drug- or cytokine-induced cell death on top of this spontaneous cell death. As a consequence many functional studies have been either conducted in vivo in mouse models, which has its limits due to ethical and economical considerations, or in intestinal epithelium-derived tumor cells. However, tumor cells have been selected to survive and to grow, which makes them difficult to compare with mature, differentiated and apoptosis-sensitive intestinal epithelial cells. For example, most cell lines, including colorectal tumor cell lines, are intrinsically insensitive to TNFα, whereas primary intestinal epithelial cells are among the most sensitive cells in our body [8].

Thus, a model system more closely related to the in vivo situation is required to study cell death in intestinal epithelial cells in vitro. Work pioneered by the research groups of Hans Clevers

and Toshiro Sato, and subsequently adapted by others has lead to the development of 3D-cultures, which allow the growth of intestinal organoids from isolated intestinal crypts or intestinal stem cells [12–14]. These organoids show differentiation of stem cells into all mature epithelial cell types also observed in vivo, including mature absorptive epithelial cells, goblet cells and Paneth cells. Importantly, these organoids show comparable responses and sensitivities to apoptosis-inducing treatments as intestinal epithelial cells in vivo [8]. Thus, they provide an ideal tool to study mechanisms of and sensitivity to apoptosis induction in primary intestinal epithelial cells in an ex vivo model system with high relevance to the in vivo situation. Furthermore, by the use of intestinal crypts from various genetically modified mice the relevance of given gene products in the regulation of cell death in intestinal epithelial cells can be investigated [8]. This protocol describes the isolation of intestinal crypts form murine intestinal tissue, their outgrowth into intestinal organoids, and the quantitative assessment of cell death induction.

2 Materials

1. 1× Dulbecco's PBS (pH 7.4): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 in ddH_2O (filter sterilized). This is referred to as PBS in the Subheading 3.
2. N2 chemically defined serum free supplement (Invitrogen): Aliquot and store at −20 °C.
3. B27 chemically defined serum free supplement (Invitrogen): Aliquot and store at −20 °C.
4. Basal crypt medium: Advanced DMEM/F12 (Sigma), 0.1 % bovine serum albumin (BSA)
   2 mM L-glutamine 10 mM HEPES, 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), 20 µg/mL nystatin, 1 mM *N*-acetyl cysteine (Sigma). Store at −20 °C in 48.5 mL aliquots. After thawing one aliquot, add 500 µL of 1× B27 supplement and 1 mL of 1× N2 supplement and store at 4 °C up to four weeks.
5. Complete crypt medium: Basal Crypt medium, freshly add 100 ng/mL murine EGF, 100 ng/mL murine Noggin, 500 ng/mL human R-spondin-1 (*see* **Note 1**).
6. Matrigel (growth factor reduced) (BD Biosciences): Thaw on ice overnight, store at −20 °C in 400 µL aliquots.
7. Human R-Spondin 1 (Preprotec): Dissolve in 1× Dulbecco's PBS, 0.1 % BSA at 100 µg/mL, store aliquots at −80 °C.
8. Murine Noggin (Preprotec): Dissolve in 1× Dulbecco's PBS, 0.1 % BSA at 100 µg/mL, store aliquots at −80 °C.

9. Murine EGF (Preprotec): Dissolve in 1× Dulbecco's PBS, 0.1 % BSA at 100 µg/mL, store aliquots at −80 °C.
10. MTT stock solution (Sigma): 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide) in ddH_2O (sterile filter), store at 4 °C
11. SDS-solution: 2 % sodium docecyl sulfate in ddH_2O.
12. Dimethylsulfoxide (DMSO).
13. Plain, clean surface to work with intestine (*see* **Note 2**).
14. 50 mL conical tubes, some with holes drilled in the lid (*see* **Note 3**).
15. Blunt end scissors.
16. Microscope (Minimum magnification: we use a 4× objective throughout. For detailed images we use up to 20× objectives).
17. 100 µm mesh cell strainer.
18. Coverslips (24 mm × 24 mm, 0.13–0.16 mm thickness).
19. 96-well flat-bottom plates.
20. 15 mL conical tubes.
21. p1000 pipette and tips.

3 Methods

3.1 Crypt Isolation

Keep crypts and solutions on ice at all stages of the procedure, unless stated otherwise.

1. Sacrifice a mouse (*see* **Note 4**), cut the small intestine (distal from stomach proximal from cecum) and store it in cold PBS until use. At this point, have Matrigel aliquot(s) thawed on ice.
2. Cut intestine open longitudinally and flatten it (*see* Fig. 1a, b) on a plain, clean surface with the cut (luminal) side facing up (*see* **Note 2**).
3. Use a coverslip to mechanically remove villi by gently scraping at angle along the intestine surface (*see* Fig. 1c and **Note 5**).
4. Cut the intestine in to pieces of 2–3 cm lengths and wash 3× with 20 mL PBS in a 50 mL conical tube by shaking vigorously for at least 10 s (*see* **Note 3**).
5. Incubate the intestinal tissue in 2 mM EDTA in 30 mL PBS in a 50 mL conical tube for 30 min at 4 °C, on an overhead rotator. Make sure that your PBS does not contain any Ca^{2+} or Mg^{2+} ions, which will saturate and inactivate EDTA.
6. Replace EDTA/PBS with normal PBS and gently shake (4–6×). Inspect the supernatant using a microscope (e.g., in a 96-well plate) (*see* Fig. 2a).

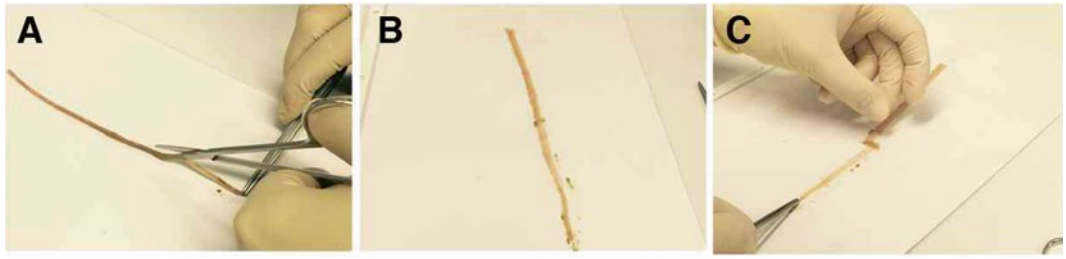

Fig. 1 Preparation of small intestine and villus removal during crypt isolation (*see* steps **2** and **3**). (**a**) Use scissors to open the small intestine longitudinally. The best technique is to put the lower part of the opened scissors into the intestinal tube and slide through the whole organ while fixing the beginning to the preparation surface with forceps. (**b**) Spread the intestine on a flat surface with the luminal side facing upwards. (**c**) Use a coverslip to physically remove villous structures by sliding it alongside the intestine

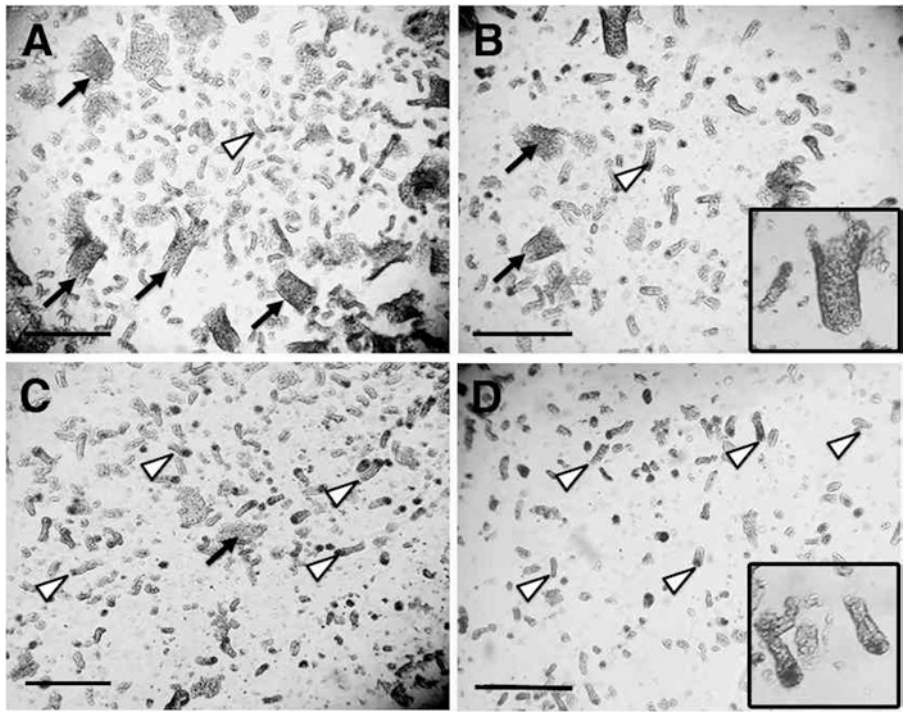

Fig. 2 Differential isolation of intestinal crypts by shaking and subsequent centrifugation step (*see* step **6–10**). (**a**) Villus-rich supernatant of EDTA-treated intestinal tissue pieces after initial shaking (*see* step **6**). (**b**) Supernatant after a second shaking step (*see* step **7**) with still some villus contained in the fraction (inlay: villus in detail). (**c**) Supernatant after a third shaking step (*see* step **7**). Only some villus fragments can be detected. (**d**) Final crypt-enriched fraction (*see* steps **9** and **10**) after straining and single cell removal by centrifugation (inlay: crypts in detail). *White arrowheads*: crypts (exemplary). *Black arrows*: villi (exemplary). Scale bar = 300 μm

7. If there are many villi and only few crypts (a threshold rate of 50 crypts to 1 villi can be considered as satisfactory for continuing), transfer supernatant to a new tube, add 20 mL of PBS to your tissue pieces, to shake them again (10–20×), this time more vigorously.
8. If the "contamination" with villi is still high, repeat **step 7** several times still the ratio crypt/villus is optimal, each time increasing the quantity and intensity of shaking. Use the microscope to decide if any additional shaking step is necessary (*see* Fig. 2b–d). Keep each fraction of your isolation on ice until you know which one you will proceed with.
9. Decide, which fraction has the best crypt/villus ratio and contains the highest number of intact crypts. Proceed with this fraction. Strain the fraction through a 100 μm mesh cell strainer (*see* **Note 6**). This will retain residual villi. Centrifuge the crypt enriched fraction at 100 × *g* for 5 min.
10. Decant supernatant and add 10 mL PBS. Transfer into 15 mL conical tube and centrifuge at 60 × *g* for 3 min. This will remove single cells.
11. Decant supernatant and resuspend pellet in 4–6 mL PBS. Use 25 μL for counting of crypt number in a flat bottom 96-well plate (*see* **Note 7**). Only count nice cup-shaped intact crypts, and calculate amount of crypts/mL (by multiplication with 40). If the quantity is too high for counting, use PBS to further dilute the resuspended crypts.
12. Take the appropriate volume with the total number of crypts to be seeded (100–300 crypts/well in a flat-bottom 96-well plate) and centrifuge at 100 × *g*, 5 min.
13. Completely remove supernatant and carefully resuspend crypts in Matrigel (we use 100–300 crypts in 8 μL of Matrigel) (*see* **Note 8**). Avoid creating air bubbles during resuspension. Seed crypts in a flat bottom 96-well plate (8 μL/well) by applying a droplet of crypt-containing Matrigel into the middle of each well. Be careful that the droplet does not get in contact with the wall of the well.
14. Let Matrigel polymerize at 37 °C in the incubator (15–20 min). Bring crypt medium to room temperature (*see* **Note 9**).
15. Add 80 μL of crypt medium to each well. Let crypts grow into organoids for 3 days at 37 °C and 5 % CO_2 in a humidified cell culture incubator (*see* Fig. 3).

3.2 Apoptosis Induction and Detection

1. Treat organoids as desired (*see* **Note 9**). Keep several wells untreated as control and treat at least one well with staurosporine (30 μM) as a positive control for cell death induction. This

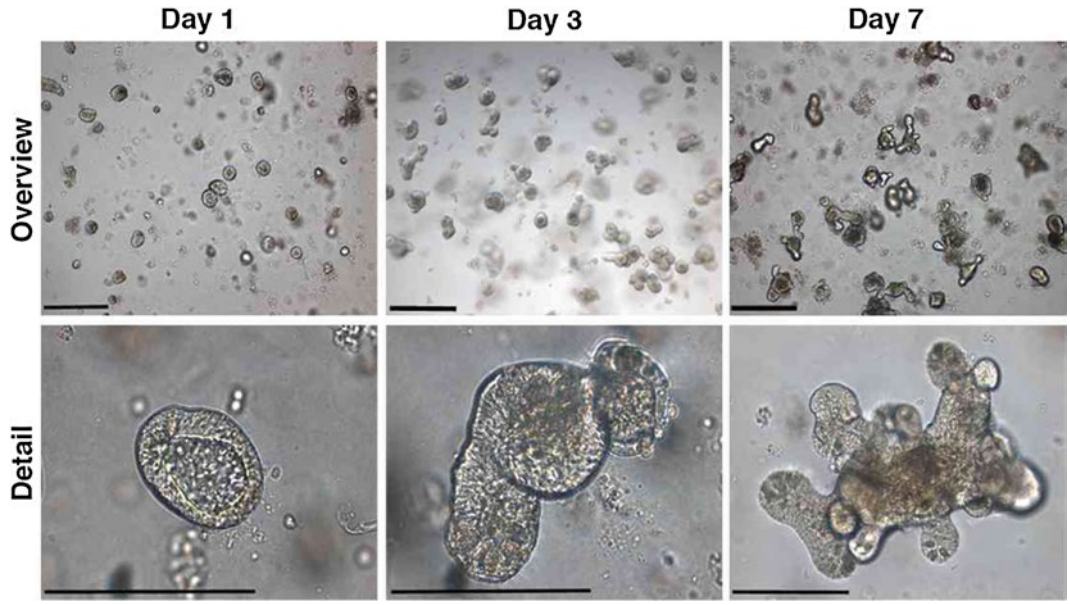

Fig. 3 Typical development of ex vivo cultured intestinal organoids. After one day of culture, crypts have closed to spheroids with a round, polar structure. At day 3 organoids form buds, crypt-like compartments, that contain Paneth cells, stem cells, and proliferating cells. Dead cells start to accumulate in the lumen of the shared central compartment, which consists of differentiated enterocytes. After 7 days of culture, a fully grown enteroid has formed with multiple crypt-like structures that feed into one shared central villus-like domain. Scale bar (overview) = 300 μm. Scale bar (detail) = 150 μm

will be needed later on for background subtraction (*see* Subheading 3.2, **step 6**).

2. Add 8 μL of MTT solution to each well (final concentration 500 μg/mL) and incubate for 1 h at 37 °C. Check for purple organoid staining with a microscope from time to time (*see* Fig. 4a–c).
3. Remove MTT containing medium completely and add 20 μL of a 2 % SDS solution. Incubate for 1 h at 37 °C to dissolve Matrigel (*see* **Note 10**). Occasionally agitate the plate gently.
4. Add 80 μL of DMSO into each well and incubate at 37 °C for 1 h to solubilize the metabolic product formazan.
5. Use a microtiter plate reader to determine absorbance at 562 nm. Subtract staurosporine-treated organoids (100 % death control) as background.
6. Use the following formula on background-subtracted OD values to calculate the percentage of dead cells:

$$\%\ \text{dead cells} = \left[1 - \frac{\text{OD}_{562}\left(\text{sample}\right)}{\text{OD}_{562}\left(\text{control}\right)}\right] \times 100\,.$$

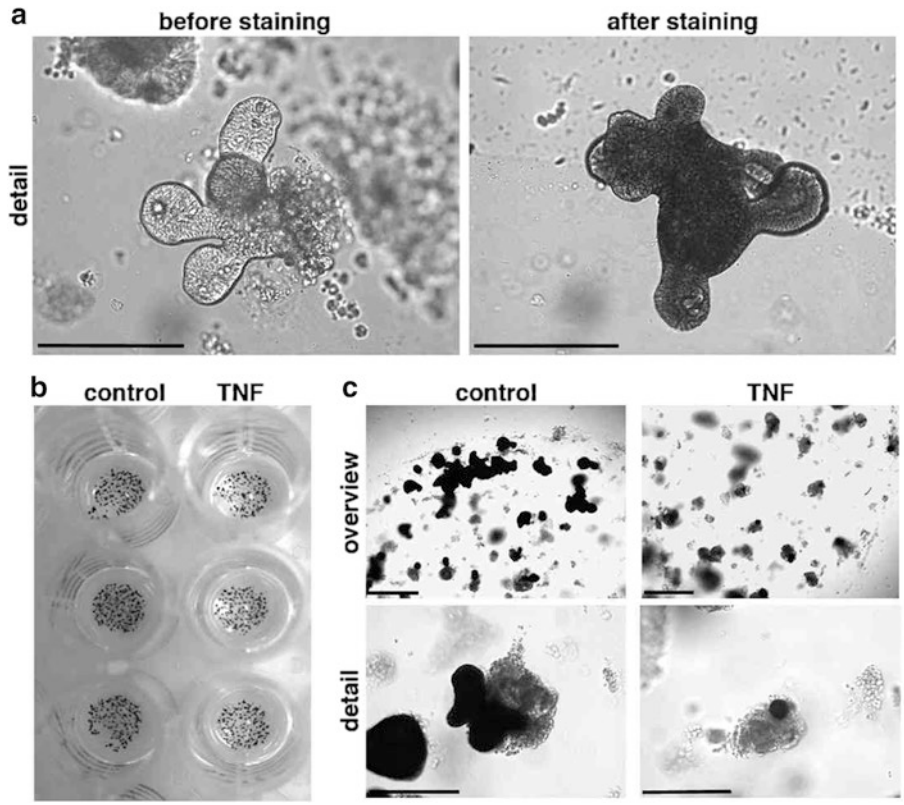

Fig. 4 Assessment of MTT reduction in intestinal organoids (*see* **step 17**). (**a**) Exemplary image of an organoid before the MTT staining process (*left*) and after 20 min of incubation at 37 °C (*right*). (**b**) Macroscopic image of stained organoids within a 96-well plate after control treatment or treatment with 30 ng/mL TNF. (**c**) Microscopic images of control or TNF-treated organoids after MTT staining. Scale bar (overview) = 300 μm. Scale bar (detail) = 150 μm

4 Notes

1. Some suppliers already offer ready-to-use complete crypt medium. These are in some cases even cheaper than buying the single components.
2. For a plain, clean surface, we use the lid of a styrofoam box covered with aluminum foil. For cutting the intestine, it is best to use scissors that have blunt tips to prevent piercing through the tissue. This enables you to cut the intestine open longitudinally in one sliding motion, without repeatedly trying to find the opening (*see* Fig. 1a).
3. To easily remove the PBS from the tissue pieces, we prepared a lid of a 50 mL conical tube with several holes drilled through it. These holes prevent the tissue pieces from being discarded while the buffer can go through. Just turn the conical tube

with the "special lid" around in a sink and apply some shaking movements to remove the PBS.

4. Commonly we use C57BL/6 mice, but other mouse strains also work fine. We usually do not use mice that are older than 16 weeks. The use of mice between 3 and 12 weeks of age gives a satisfactory yield of intestinal crypts.
5. Note 5 Using a coverslip will prevent you from applying too much pressure on the intestine, which will result in loss of crypts and a poor yield in the end. Just do not press so much that the coverslip breaks (*see* Fig. 1c).
6. If your fraction is already highly enriched in crypts and hardly contains any villi, you can skip the cell straining step at this point and proceed directly to the next step.
7. Crypts are too big to fit into a classical Neubauer cell counting chamber. We therefore count the crypts by pipetting 25 μL of the crypt-enriched supernatant into a flat bottom 96-well plate. The concentration of crypts/mL can easily be calculated by multiplication of (number of crypts in 25 μL) × 40.
8. Decanting is not sufficient to remove the supernatant completely. For obtaining a drier pellet, we usually first decant the supernatant and then remove the rest with a p1000 pipette. Be very careful to not disturb the pellet.
9. Make sure that the crypt medium is not cold, when it is added to the already solidified Matrigel droplets. If the medium is too cold, it will liquefy the Matrigel. As final volume, use the volume of Matrigel + medium. We usually treat the organoids 16 h or overnight, and assess cell death after that. To get a good idea about the kinetics and extend of cell death induction we recommend to start with a dose response of a standard cell death inducer (e.g., cisplatin, actinomycin D) and overnight incubation.
10. Do not cool the microtiter plate at this step. Otherwise this will cause the SDS to precipitate. If the Matrigel is already dissolved, you can see the purple stained organoids floating around under the microscope (and even by eye).

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