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A novel morphometry system automatically assessing the growth and regeneration of intestinal organoids

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ABSTRACT

As compared with 2D cell line cultures, 3D intestinal organoids are better at maximally recapitulating the physiological features of stem cells *in vivo*. However, the complex 3D structure is an obstacle which must be objectively and automatically evaluated to assess colony growth and regeneration. Meanwhile, no internal standard currently exists for evaluating the size of heterogeneities in organoids or defining those regenerating colonies. Herein, we developed a simple morphometry system to image MTT-stained organoids. The growth curve of organoids can be automatically generated based upon analyzing the integrated optical density using software. Referencing the definition standards of *in vivo* regenerating crypts, the perimeters of crypts cultured 24 h after seeding were selected as an "Organoid Unit" to further evaluate colony survival rate and colony size heterogeneities after exposure to varying doses of irradiation. Moreover, the morphometry-based quantification data collected confirmed other findings associated with radiation sensitizing effects of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) inhibitor and the radiation protective effect of IL-22. In summary, the novel organoid morphometry system combined with a new internal reference is a practical means for standardizing assessment of growth, survival and regeneration of intestinal organoid colonies. This method has promise to facilitate drug screens in intestinal and other organoid systems.

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1. Introduction

Intestinal epithelium is a good model for a stem cell study due to its high turnover speed and compartmentalized structures. Methods to develop the epithelium properly for study with stem cells include removal of all clonogenic cells. Some crypts are eradicated after exposure to cytotoxic agents including radiation and chemotherapy, both of which have been utilized as a tool to investigate the intestinal stem cells (ISC) in a natural environment for over 40 years [1–3]. Compared with intestinal cell lines, organoids cultured in 3D matrigel are more apt to recapitulate the native physiological features of crypt-villi units, representing a powerful tool for modeling diseases and investigating stem cell biology in the dish [4–7]. However, it is difficult to objectively quantify cell growth and regeneration in *ex vivo* 3D organoid

culture due in part to multiple issues with high Z-stack, dead cell mixtures and high heterogeneities in growth rate and colony size. Meanwhile, in analyzing tissue regeneration, the size of the proliferating population of normal crypts can be associated with the regenerating crypt with most similar proliferating cells as an evaluative measure [8,9]. While these methods prove promising, there remains no consensus as to what parameters are taken as an internal control to evaluate the survival and regeneration in *ex vivo* organoid systems [10–14]. Here, we developed a simple morphometry system to objectively and automatically evaluate tetrazolium component (MTT)-stained organoids.

2. Material and methods

2.1. Crypt isolation and culture

Mouse Intestinal crypt-villus units were isolated and stained as previously described [15]. Briefly, the intestines were obtained

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from 8-to 10-week-old C57BL/6 mice and the fecal matter was flushed with ice-cold DPBS (Hyclone). After filleting open by cutting longitudinally through the lumen, the intestine was rinsed thoroughly in ice-cold DPBS. The intestine was cut into 2–3 mm pieces and incubated in the dissociation buffer (DPBS with 30 mM EDTA) for 20 min on ice. After replacing dissociation buffer with DPBS, pieces of intestine were incubated in a 50 ml tube for 15 min on ice. To release epithelium from basement membrane, the tube was shaken for about 30–60s. The mixture was passed through a 70 μ m cell strainer, and crypts were isolated and purified through centrifugation at 4 °C. Then the crypts, mixed with Matrigel (Corning), were seeded in 96-well flat-bottom plates (10 μ l/well). The plates were incubated at 37 °C for 17 min. And the culture medium, which composed with Advanced DMEM/F12 (Gibco), 1 $\times$ penicillin–streptomycin (Beyotime), 10 mM HEPES (Gibco), 1 $\times$ GlutaMAX (Gibco), 500 μ M N-Acetyl-L-cysteine (SIGMA), 1 $\times$ N2 (Gibco), 1 $\times$ B27 (Gibco), 1 μ g/ml Human R-Spondin 1 (R&D), 50 ng/ml Murine EGF (Peprotech), 100 ng/ml Murine Noggin (Peprotech) and 10 μ M Y-27632 (SIGMA), were added into wells (100 μ l/well). The growth factors (R-spondin 1, EGF and Noggin) were added every other day and the entire culture medium was replaced after 4 days of seeding. Y-27632 was then withdrawn and not included in subsequent culture maintenance.

2.2. Colony irradiation and treatment

After 24 h of culture, organoids were exposed at a dose rate of 0.9 Gy/min using an X-ray irradiator (X-RAD 320). In some experiments, the inhibitors and mIL-22 were administered 15 min and 1 h respectively before irradiation. The survival cells were stained with MTT. MTT (BBI) solution was added to the culture medium to a final concentration of 500 μ g/ml. After incubation for 40 min at 37 °C, 5% CO₂, the medium was replaced with PBS (100 μ l/well).

2.3. Image capture and data collection

The culture plates were placed on an electroluminescent panel with lids removed. The images of the stained organoids in the wells of the plate were captured by the iPhone 6S, which was set 5 cm above the plate and equipped with ZEISS lens (ZEISS Mutar™ 2.0x Asph Telephoto lens Exceptional edge to edge contrast and virtually no distortion or chromatic aberration ExoLens PRO with Optics by ZEISS <https://exolens.com/product/exolens-with-optics-by-zeiss-telephoto-2x/>). The parameters (area, IOD, number of organoids, etc.) of each image were generated by the software (Image-Pro Plus 6.0) automatically. Each value was presented as a mean of the tests of at least 3 wells.

2.4. Statistical analysis

All statistical analyses were performed using the Graph Pad Prism software (Ver. 6.01) (GraphPad Software, San Diego, CA, USA). Results were expressed as mean $\pm$ standard deviation (SD). Statistical significance was tested by one-way ANOVA for multiple comparisons or T-test for two group comparisons. $P < 0.05$ was taken as statistically significant.

3. Results

3.1. A new morphometry system for objectively and automatically evaluating organoid size

We hypothesized that the MTT-stained organoids could be scanned and quantified as a dot blot. Thus, we developed an imaging system with a Zeiss lens, Phone Camera and

electroluminescent panel (Fig. 1C) capable of capturing images of individual wells. This imaging system avoided the collusion of images seen with Z-stacking and tallied all live colonies in a single image (Fig. 1D). Moreover, a series of parameters, such as area and integrated optical density (IOD), were easily analyzed using software Image-Pro Plus 6.0 (Fig. 1E). Stained dead cells did not interfere with the live cell colony images, and were normalized with the background stain (Fig. 1E). The data collected relating to the tested parameters were found to be consistent with the growth state of organoids (Fig. 2A, B, C). Moreover, the irradiated organoids containing different amounts of dead cells inside may have similar surface area but significant different IOD, which makes IOD a more apt parameters to evaluate the growth state of organoids (Supplemental Fig. 1).

3.2. The new morphometry system can objectively evaluate the growth of organoids

We further tested the likelihood of accurate organoid culture growth curve plotting. As shown in Fig. 2C and D, although the initial sizes of organoids were not equivalent (Fig. 2A), when the parameters of area or IOD of single organoids were averaged, the organoid sizes gradually rose in conjunction with culture times (Fig. 2B). Growth curves were then assessed on exposure to radiation. Compared with the non-irradiated control group, a single exposure to 3Gy irradiation induced instant organoid growth retardation (Fig. 2E and F). Recovery of colony growth rates was significantly delayed when the irradiation doses increased to 7Gy (Fig. 2E and F). In summary, our morphometry system effectively evaluated the organoid growth under both normal and pathological conditions.

3.3. The new morphometry system can evaluate colony heterogeneities and regeneration by “Organoid Unit”

The survival microcolony on day 3.5 to day 4 after exposure to lethal doses of irradiation is defined by a crypt containing similar or exceeding amount of proliferating cells compared with normal crypts [13,16]. Usually, over 5–6 BrdU incorporation-positive cells in a crypt have been taken as internal reference [9,12,16], seemingly like a “crypt unit”. As shown in Fig. 3A, approximately 29.7% of crypts survived for regeneration after 10Gy X-ray irradiation. However, no internal reference existed to eliminate those colonies with too few surviving cells nor to properly evaluate the heterogeneities of the regenerating organoids (Fig. 3B). Utilizing the area of the crypts immediately inoculated as a reference unit, we determined that the truncation of crypts and the mixed villi fragments resulted in high variations, similar to the heterogeneities noted earlier (Fig. 3C). Moreover, the mixed villous cells or truncated crypts without a stem cell zone died after a 24 h culture, whereas nearly all survival colonies studied grew into isolated passage-able organoids (Figs. 2A and 3C). Furthermore, the estimated areas of those colonies cultured for 24 h were more equivalent compared with those crypts seeded and cultured for 48 h (Fig. 3D–F). Thus, we defined the measurable perimeter of an organoid cultured for 24 h as an “Organoid Unit” (OU). Use of this OU aided in defining survival colonies and evaluating the heterogeneity of colony size.

We then used OU to evaluate the survival colonies in the radiation model. Consistently, most colonies survived after exposure to a low dose of irradiation, larger than one OU. It was found that tiny live cell masses were generated after exposure to high doses of irradiation, which were filtered out in imaging by setting the threshold of one OU (Fig. 3G). The size heterogeneity of the survival colonies was easily and automatically defined by calculating the

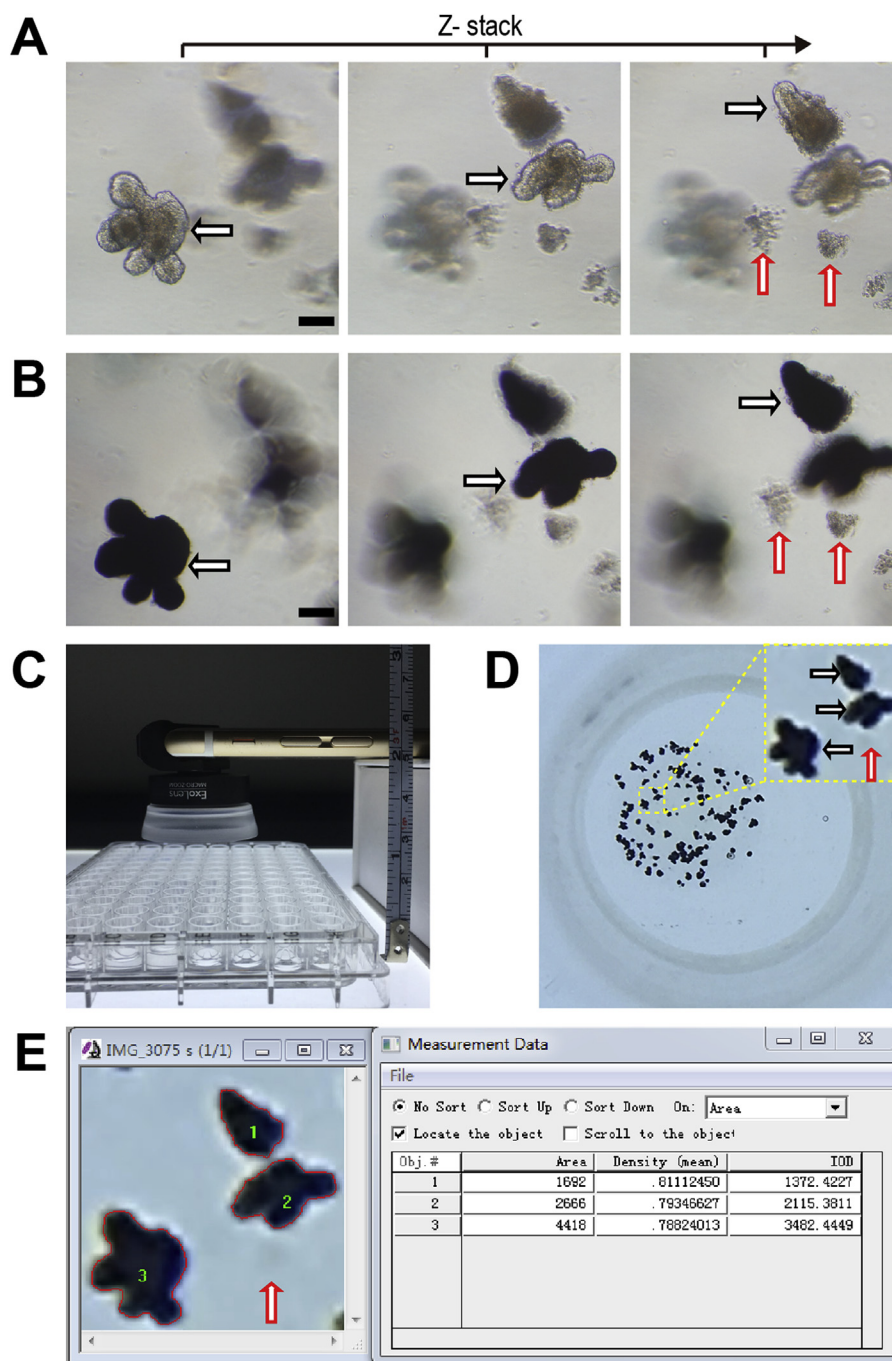


Fig. 1. A new imaging system for evaluating live organoids objectively and automatically. Crypts inoculated onto 96 well plates were cultured for 4 days and images were captured before and after MTT staining. Data shown are representative of six independent experiments. (A) Images of live organoids with continuous Z-stack were captured under an inverted microscope; (B) The same organoids as in A were stained with MTT and the images were captured under a light microscope. Black arrow heads indicate the organoids; red arrow heads indicate dead cells; (C) A new macrophotography imaging system comprises a Zeiss lens, Phone Camera and electroluminescent panel. (D) Images of a single organoids representing all MTT-stained organoids with different Z-stack; black arrow heads indicate the single organoids; red arrow head indicates the organoids were the same organoids shown in (B); (E) Automatic analysis of the parameters of stained organoids including area and IOD by Image-Pro Plus 6.0; the area and grayscale of the stained organoids were digitalized. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

count of large (>40U), middle (2-40U) and small colonies (1-20U) (Fig. 3H). Furthermore, size heterogeneity was used as an indicator of the regeneration ability of organoids after X-ray radiation (Fig. 3H). The survival rate dramatically decreased by 5–6Gy radiation (Fig. 3H and I) and the dose of LD50 was about 6Gy (Fig. 3I). In summary, our morphometry system evaluated colony survival and regeneration using the perimeter of an organoid cultured for 24 h as an “Organoid Unit”.

3.4. The new morphometry system can objectively evaluate the effect of radiation sensitizing or protective agents on ISC survival and regeneration

The effect of several small molecule inhibitors of ATM and ATR, which inhibit DNA damage response [17,18], on irradiation-induced organoid damage and regeneration were tested by our morphometry system. As we expected, ATM inhibitor (KU-60019, CP-466722)

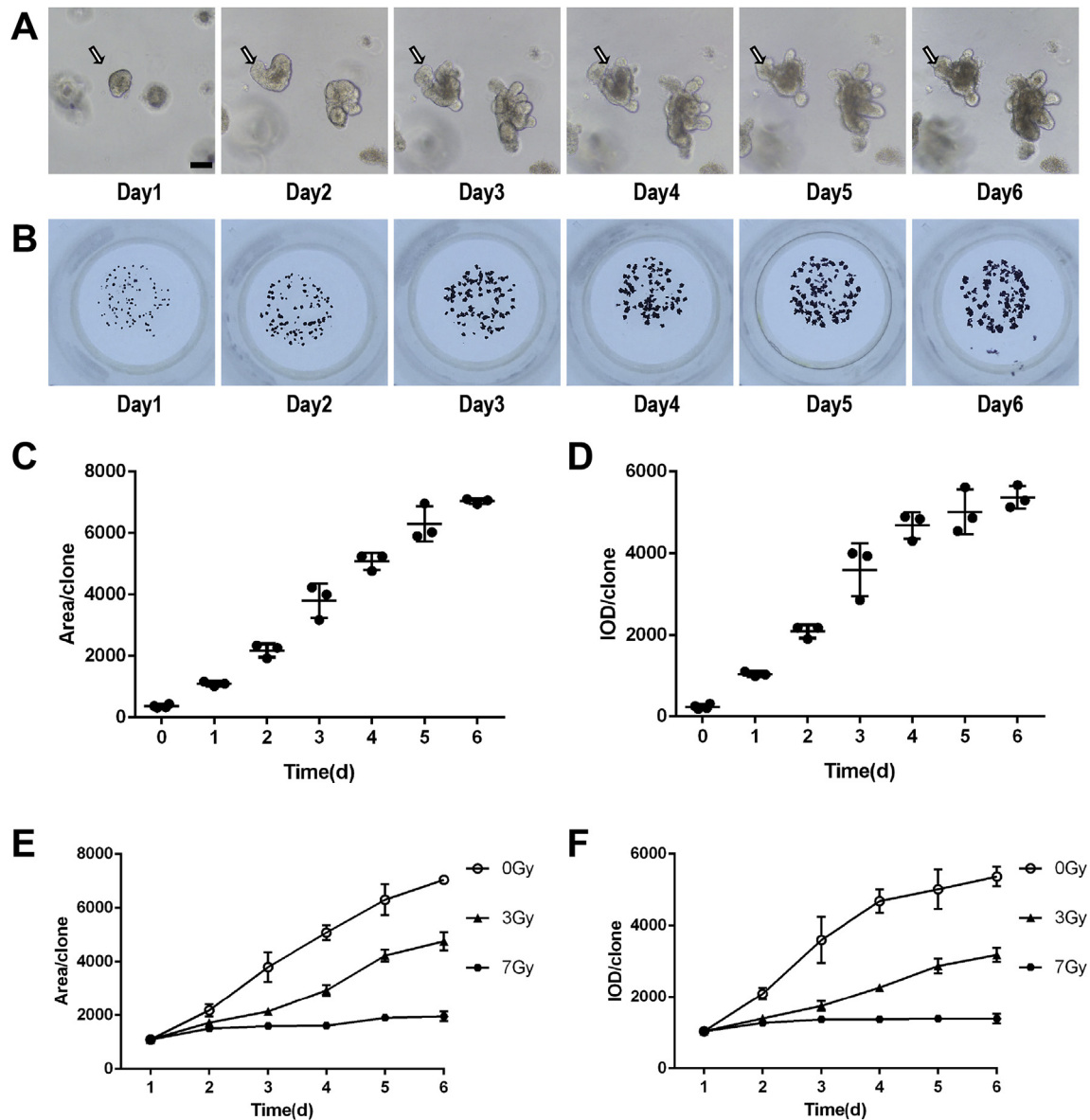


Fig. 2. Evaluation of organoid growth using the new imaging system. Crypts were inoculated onto 96 well plates with at least 3 wells per time point for each test group. The organoids from the same mice were stained on a different day and images were captured on the final day. For the irradiation model, the organoids on the same plate were exposed to different X-ray doses with the aid of lead sheets to selectively block the radiation of certain wells from the radiation at some doses. Those organoids were then stained on the given days together. Data were represented by three independent experiments. (A) Growth of crypt-derived organoids, arrow head indicates the same organoid on different days; (B) Macrophotography images of MTT-stained organoids cultured from Day 1 to Day 6; (C) Growth curve plotted based on colony area; (D) Growth curve plotted based on IOD level; (E) The effects of 3 and 7 Gy X-ray irradiation on the growth of organoids based on colony area; (F) The effects of 3 and 7 Gy X-ray irradiation on the growth of organoids based on IOD level.

and ATR inhibitor (AZD6738, VE-822) [19–24] significantly decreased the survival number and sizes of organoids as compared with the vehicle group (Fig. 4A–D). We also tested a known radiation protector IL-22 [12], which significantly increased the size and number of survival organoid colonies after irradiation (Fig. 4E–G).

4. Discussion

Although the number of theoretically seeded crypts could be quantified using the relative MTT staining [25], the organoid surface area was instead measured manually in an effort to identify the heterogeneities of organoid sizes [10–13], proving laborious and subjective. In the present study, our simple macrophotography

system transformed the complex 3D imaging into easily-quantified dot blot imaging, surmounting the main obstacles for organoid parameter measurement.

This system presents several advantages for evaluating the colony growth and survival. Although many researchers have their own protocols for isolating and culturing organoids [5,15,26–29], which may lead to different growth rate of organoids and radiation sensitivity, they still can set up their own internal control. Moreover, since the growth can be evaluated based on the average of single organoids, the heterogeneities in intrinsic expansion ability of single crypt and the different seeding number in the wells can be normalized, which make it easy for high-throughput screening. In addition, the macrophotography system is relatively cheap and even do not need microscope, which may be feasible for

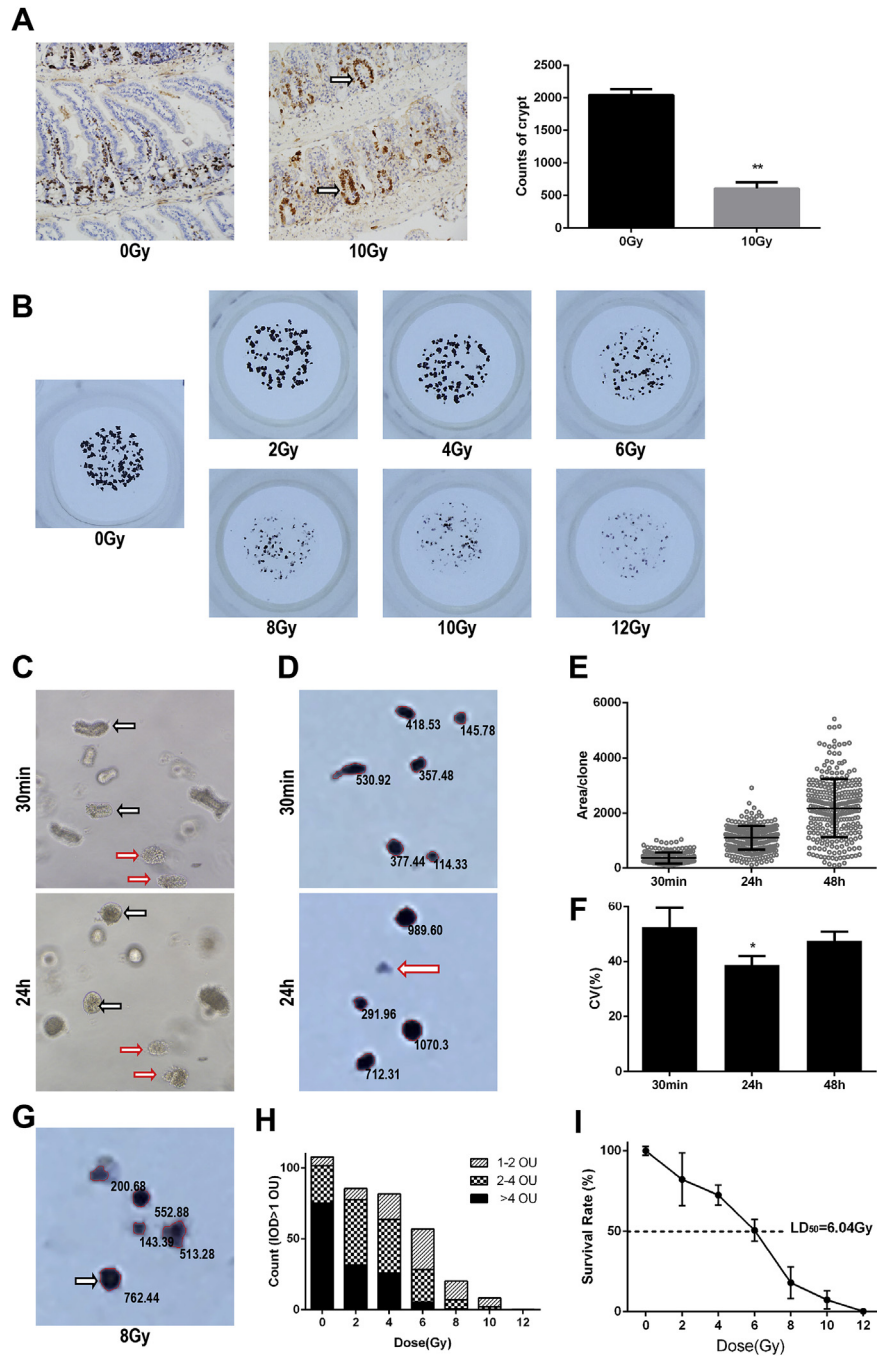


Fig. 3. Definition of organoid unit for evaluation of survival colonies and size heterogeneities. (A) IHC staining shows the BrdU labeled cells in mouse intestines on the fourth day after 10Gy X ray radiation in both normal and radiated crypts. The survival rate of crypts was evaluated by the number of micro-colonies containing more than 6 BrdU positive cells ($n = 4$, $**p < 0.01$); (B) Dose effect of X-ray radiation on the survival of organoids; crypts inoculated onto 96 well plates were cultured for 48 h and then exposed to different doses of irradiation. The organoids were stained and images were captured on the fourth day after irradiation. Experiments were performed in triplicate; (C) Representative images of the crypts 30min and 24 h after inoculation; black arrows indicate the clone-genic crypts and red arrows indicate the mixed villous cells and truncated crypts without clone-genic ability; (D) Representative macrophotography images of the crypts 30 min and 24 h after inoculation with IOD values indicated for each crypt; (E) Comparison of the sizes of organoids at 30 min, 24 h and 48 h after inoculation ($n_{30min} = 189$ colonies, $n_{24h} = 276$ colonies, $n_{48h} = 278$ colonies); (F) The coefficient of variation (CV) ($n = 4$, $*p < 0.05$). The average of IOD value of 24 h culture was taken as 1 organoid unit as internal control since the CV at 24 h after was the lowest; (G) Representative macrophotography images of the colonies on the fourth day after 8Gy irradiation; and arrow indicates the colony with an IOD value higher than 10OU (IOD = 570 in given experiment); (H) Effects of different doses of radiation on the number and size of the survival colonies, which shows the heterogeneities of survival colonies based on macrophotography image and OU reference ($n = 3$); (I) LD 50 of irradiated intestinal organoids ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

laboratories worldwide.

There is a high percentage of people suffering gastrointestinal symptoms after abdominal and/or pelvic radiotherapy [30]. An objective and automatic evaluation system is in urgent demand

for screening radiation sensitizing agents or radiation protective agents based on ISC survival [31]. It has become attractive in cancer therapy to inhibit DNA damage response using small molecule inhibitors of ATM and ATR [17,18]. Most studies focus on the radiation

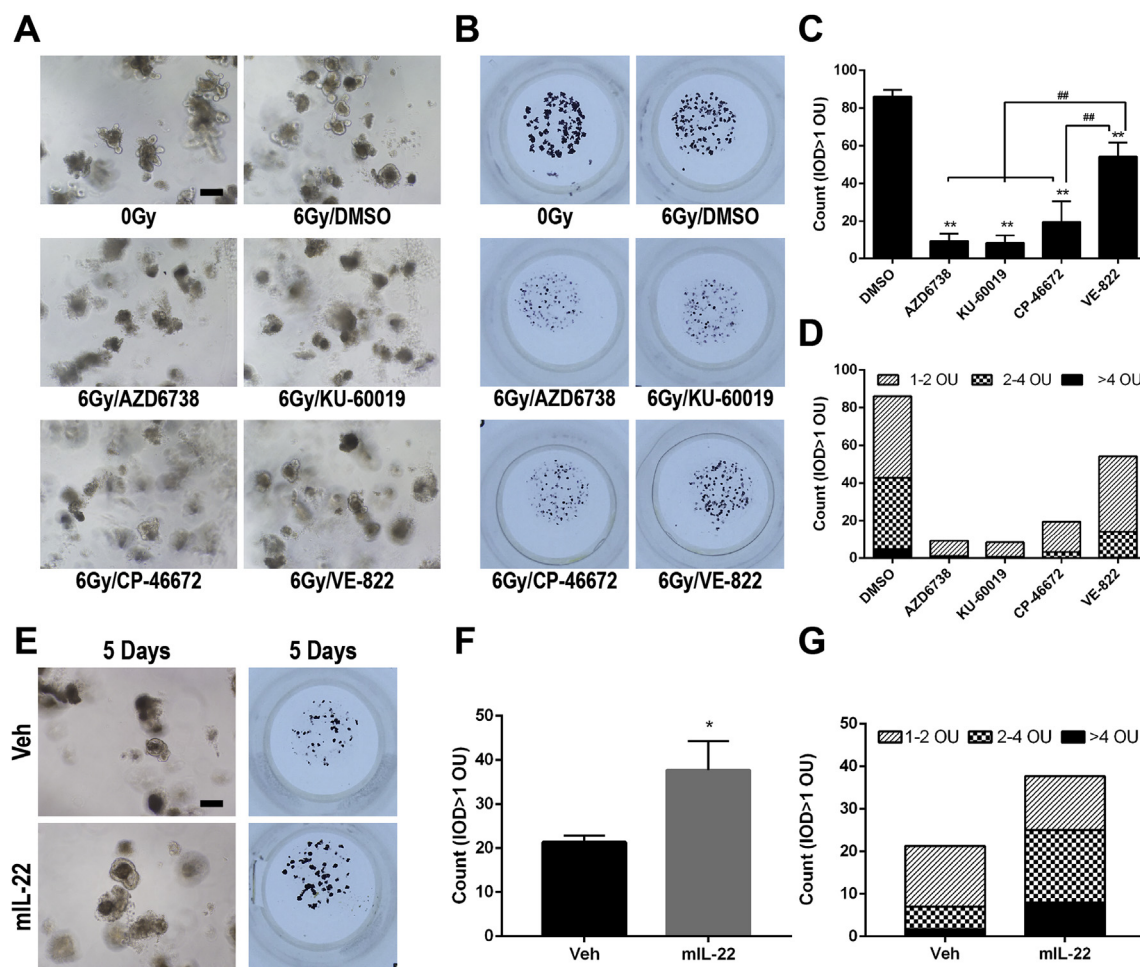


Fig. 4. Assessment of the effects of radiation sensitizing agents and radiation protective agents on organoids using the macrophotography image system based on a reference organoid unit. Crypts were inoculated onto 96 well plates with 5 wells for each group; after being cultured for 48 h, the organoids were incubated with vehicle, KU-60019 (3 μ M), CP-46672 (3 μ M), AZD6738 (6 μ M), and VE-822 (0.1 μ M), respectively, for 2 h prior to radiation (6 Gy). (A) Images of the organoids on the fourth day after X-ray irradiation and treatment with different sensitizing drugs under an inverted microscope; (B) Representative macrophotography images of the organoids on the fourth day after X-ray irradiation and treatment with different sensitizing drugs; (C) Comparison of survival organoids treated with different sensitizing drugs treatment quantified based on macrophotography images and organoid unit; (D) Size heterogeneities of the survival organoids treated with different radiation sensitizing drugs assessed based on organoid units; (E) Images of the organoids on the fifth day after 6 Gy X-ray irradiation with and without mIL-22 (2 ng/ml) treatment; (F) Quantitative assessment of the effect of mIL-22 on the survival organoids; (G) Quantitative assessment of the effect of mIL-22 on the organoid size heterogeneity (n = 3, *p < 0.05, **p < 0.01, ###p < 0.01, Bar = 100 μ m).

sensitize effect of these inhibitors on cancer cells. Herein, we emphasize the side effect on normal stem cells. It is worth noting that without MTT staining and IOD counting, the area counting of live organoids in bright field in previous studies [10,12–14] could not establish a clear boundary of regenerating organoids and ignored the different signal densities caused by large amount of dead cells inside the organoids (Supplemental Fig. 1). Thus, our colony imaging method combined with OU as an internal control was a much more practical tool to evaluate the radiation sensitizing and protective drugs.

In summary, based on the new macrophotography system and recommended organoid-unit, we provide a feasible and fundamental method for evaluating growth, survival and regeneration of intestinal organoids. It is expected that this method will be applicable for studies involving other tissue organoids and help to facilitate an organoid-based drug screen.

Conflicts of interest

The authors declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2018.10.181>.

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