A fast and simple fluorometric method to detect cell death in 3D intestinal organoids

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ABSTRACT
Organoids recapitulate the (patho)physiological processes in certain tissues and organs closer than classical cell lines. Therefore, organoid technology offers great potentials in drug development and testing, and personalized medicine. In particular, organoids can be used to study and predict drug-induced toxicity in certain tissues. However, until today few methods had been reported to analyze cell death in 3D-microtissues in a quantitative manner. Here, we describe a novel fluorometric method for the quantitative measurement of specific organoid cell death. Organoids are stained simultaneously with the cell impermeable nuclear dye propidium iodide and cell permeable Hoechst33342. While Hoechst allows in-well normalization to cell numbers, propidium iodide detects relative proportion of dead cells independent of hydrogel. Measurement and analysis time, as well as usability are drastically improved in comparison to other established methods. Parallel multiplexing of our method with established assays measuring mitochondrial activity further enhances its applicability in personalized medicine and drug discovery.

METHOD SUMMARY
We developed a fluorometric method to quantify cell death in intestinal organoids based on DNA staining for normalization and cell permeability for cell death. The method, independent of cell number over a wide range, can be used to study toxic effects of drugs on intestinal organoids or other 3D microtissues, and can be combined with photometric assessment of mitochondrial respiration.

KEYWORDS
cell death • DNA dyes • fluorescence • intestine • organoids • personalized medicine

Organoids grown from tissue-specific stem cells have become useful tools to study physiological and pathophysiological processes in an in vitro system much closer to the in vivo situation than cell lines. In 2009, Sato et al. described a method on how isolated primary intestinal crypt cells can be cultured over prolonged periods of time [1]. Today, organoids are being generated from nearly every tissue and any organism [2–4]. They are defined as organ-like structures, which self-organize in 3D. Comprised of several tissue- and organ-specific cell types, including stem cells, organoids are capable of fulfilling a variety of organ-specific functions, for example, excretion or secretion [5].

Intestinal organoids are being used to study not only basic intestinal physiology [6], but also pathophysiological processes, for example, TNFα-induced epithelial cell death during inflammatory bowel disease [7,8]. Moreover, intestinal organoids have been used to study host–pathogen interactions, for example, during Zika virus infection [9]. Importantly, intestinal organoids may also represent an unlimited source of transplantable tissue suitable for regenerative medicine. In a proof-of-principle study murine intestinal organoids were successfully transplanted into the severely damaged colon of mice suffering from inflammatory bowel disease. Organoids not only engrafted functionally into the colon but regenerated the damaged epithelium [10]. Tumor tissue-derived organoids also provide interesting tools to study tumor-specific drug responses as well as tumor diversity ex vivo, for example, in colorectal tumors [11,12]. Therefore, primary and tumor organoids are being and will be frequently employed for drug discovery [13] and toxicity testing, but also drug screening in patient-derived malignant tissue [14–16]. In this regard, patient-derived tumor organoid (PDOs) from pancreatic, prostate and gastrointestinal cancer are being used to model drug responses of patient-specific tumors in comparison to normal parental tissue [17–22]. This is of particular interest, as in gastrointestinal cancer it has been demonstrated that the parental and the PDO mutational spectra overlap up to 96%, on top of histological similarities [21].

Thus, by closely resembling the primary tumor in vivo drug testing in PDOs represents a further step towards personalized medicine [21]. Moreover, organoid-like 3D primary cell culture models are being effectively used to screen a large number of emerging oncology compounds for their cytostatic and cell death-promoting activity [23].

A significant problem of cell death screening in organoids is their 3D culture in extracellular matrix, which allows visual qualitative assessment of cell death, but impedes quantitative analysis of cell death. We have previously described the use of a modified MTT staining method to detect organoid survival, specifically, cell death in culture [24]. However, this method is strongly affected by mitochondrial respiration and only an indirect measure of cell survival, specifically, cell death. Other studies have used staining of dying organoids with propidium iodide (PI) and Hoechst33342 (Hoechst) to quantify cell death, employing complex and time-consuming high-content imaging [25].

The aim of this study was therefore to develop a simple, practical and quantitative method to study cell death...
death in organoids using PI and Hoechst. While Hoechst is being used to normalize cellularity, PI uptake serves as a measure of cell death. The normalization allows sensitive cell death detection over a wide range of cell densities. Fluorescence in 3D cultures can be quantified using a conventional plate reader. The increase of the PI signal relative to the constant Hoechst signal allows calculation of PI/H ratio under the microscope. Mean ± standard deviation; n = 3 with technical triplicates. **p ≤ 0.001; ****p < 0.0001.

BF: Brightfield; H: Hoechst; NS: Nonsignificant; PI: Propidium iodide; RFU: Relative fluorescence unit; STS: Staurosporine.

**MATERIALS & METHODS**

**Mice**

C57BL/6 wild-type mice were bred and kept in individually ventilated cages at the central animal facility of the University of Konstanz.

**Generation of intestinal organoids**

Intestinal crypts were isolated as described previously with minor changes [1,24]. In brief, the small intestine of 8–16-week-old C57BL/6 wild type mice was cut open longitudinally. Villi were removed by scraping with a microscope slide. Then, the intestine was cut into 3–4 cm pieces, washed three times with cold Ca²⁺- and Mg²⁺-free PBS, and incubated with 2 mM EDTA in PBS for 30 min at 4°C on a rotating wheel. Subsequent steps until seeding were performed on ice. Supernatant was removed and the tissue was filled up with fresh PBS. After shaking to remove residual villi, fresh PBS was replaced. This step was repeated and each fraction was checked for crypt/villus ratio under the microscope. Up to four crypt-containing fractions were pooled, filtered through a 70-μm cell strainer, centrifuged at 100 × g (3 min, 4°C) and resuspended in 5 ml PBS for crypt counting under the microscope. Numbers of crypts required for further culture were centrifuged at 80 × g (3 min, 4°C) and the pellet was resuspended in Matrigel (BD Biosciences) or in Basement Membrane Extract (BME) (Type II, R&D). A total of 200–300 crypts were seeded per well in 8 μl Matrigel or BME into a 96-well flat-bottom transparent cell culture plate (Sarstedt). Seeded crypts were incubated for 20 min at 37°C to let Matrigel and BME solidify. Then, 80 μl of complete crypt culture medium per well was added dropwise (Advanced DMEM/F12, 0.1% BSA, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM N-acetyl cysteine (Sigma), 1 × B27 supplement, 1 × N2 supplement (Gibco), 50 ng/ml mEGF, and 100 ng/ml mNoggin (Peprotech). rh-spondin-1 was added as conditioned medium of rh-spondin-1-transfected HEK 293T cells to a final volume of 25% (v/v) crypt culture medium. Organoids were cultured at 37°C in a 5% CO₂ atmosphere for 3 days before cell death induction.

**Generation of tumoroids**

Organoids from tumors (tumoroids) were generated as described previously with slight modifications [26]. Briefly, the small intestine of APCMin/+ mice was opened longitudinally. Tumors were isolated from intestinal tissue with scissors and forceps and cut into small pieces. Subsequently, tumor fragments were washed three times with ice cold Ca²⁺- and Mg²⁺-free PBS, and incubated in digestion buffer (DMEM, 2.5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 U/ml Collagenase IV, 125 μg/ml Dispase II) for 1 h at 37°C, 5% CO₂. Tissue suspensions were shaken every 15 min. After 1 h tumor fragments were allowed to settle for 1 min. Subsequently, the supernatant was harvested and centrifuged at 200 × g for 3 min at room temperature. The pellet was resuspended in 5 ml PBS and filtered through a 70 μm and a 40 μm cell strainer. After centrifugation (200 × g for
3 min) cells were resuspended in 500 μl PBS and counted. Cell numbers were adjusted to 1.5 x 10^4 cells/50 μl Matrigel/BME. Complete growth medium with only 50 ng/ml mEGF was added. The culture medium was changed every 4 days. Tumoroids were split according to their density, but in general every week. Thus, medium was removed and tumoroids were incubated in cold PBS for 1 h on ice. Subsequently, Matrigel/BME was dissociated mechanically with a pipet tip and tumoroids were resuspended in cold PBS. Then, tumoroids were centrifuged at 200 x g for 3 mins and the pellet was resuspended in TrypLE Express (Thermo Fisher) for 15 min at RT. Tumoroid fragments were then centrifuged at 350 x g for 3 min and split in a 1:4 ratio for further culture.

Culture of human intestinal organoids
Human intestinal organoids were generated and cultured as described previously [27]. Frozen organoids were thawed and cultured in a mixture of 50% basal medium containing 500 ng/ml hr-spondin-1, 50 ng/ml mEGF, 100 ng/ml mNoggin, 10 nM [Leu15]-Gastrin 1, 10 mM Nicotinamide, 500 nM A83-01 (TGFβ inhibitor), 10 μM SB202190 (p38/MAPK inhibitor), 10 μM Y-27632 (ROCK inhibitor) and 50% Wnt3A-conditioned medium. Growth medium was replenished every second to third day, and organoids were passaged weekly.

Staining of organoids with PI & Hoechst
Intestinal organoids in Matrigel/BME were stained with PI and Hoechst at a final concentration of 10 μg/ml each. Staining solution (dyes in PBS) was directly added to culturing medium after treatment. Organoids were stained for 30 min at 37°C, 5% CO₂ for subsequent analysis on the plate reader or by fluorescence microscopy.

Then, staining medium was removed and replenished with fresh phenol-red free medium before analysis.

Fluorometric quantification of specific cell death in intestinal organoids
Cell death was induced in organoids and cell lines as indicated. Before measurement, staining medium was replaced with fresh phenol-red free medium. Stained organoids still embedded in hydrogel (Matrigel/BME) were stained with PI and Hoechst at a final concentration of 10 μg/ml each. Staining solution was directly added to culturing medium after treatment. Organoids were stained for 30 min at 37°C, 5% CO₂ for subsequent analysis on the plate reader or by fluorescence microscopy.

Using PI/Hoechst ratio, treatment specific organoid cell death was calculated:

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\text{treatment-specific organoid cell death} = \left( \frac{x(\text{sample})}{z(\text{STS})} \right) \times 100 - y(\text{ut})
\]

Each sample was divided by the mean of all staurosporine (STS)-treated organoids and resulting values multiplied by 100. Then, mean of all untreated (ut) organoids was subtracted to set ut organoids to 0.

**Determination of organoid viability & specific organoid death using MTT reduction**
Organoid viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction as described in [24]. Briefly, after cell death induction MTT solution was added to the organoid culture to a final concentration of 500 μg/ml and incubated for 1 h at 37°C, 5% CO₂. Then, medium was discarded and 20 μl of 2% SDS solution in H,O was added to solubilize the hydrogel (Matrigel/BME) for 1 h at 37°C. Subsequently, 80 μl of DMSO was added and incubated for 1 h at 37°C to solubilize the reduced MTT. The optical density was then measured at 562 nm in a plate reader (Tecan M200 Pro).

Quantification of intracellular ATP
Intracellular ATP was quantified with the CellTiter-Glo® 3D cell viability assay according to manufacturer’s protocol (Promega). Briefly, after treatment medium was replaced with fresh medium containing 5% CO₂, 1 h. Then, medium was removed and cells were lysed in 100 μl pre-warmed CellTiter-Glo 3D reagent.
Then, samples were incubated for 30 min on an orbital shaker and luminescence was recorded afterwards using a plate reader (Tecan M200 Pro).

**Analysis of activated effector caspases**

Activation of effector caspases 3 and 7 was performed with CellMeter™ Live Cell Caspase 3/7 Assay Kit (blue fluorescence) according to manufacturer’s protocol (AAT Bioquest). In brief, organoids were stained with ApoBrite™ U470 Caspase 3/7 substrate for 2 h at 37°C before treatment. Then, cell death was induced and organoids stained additionally with PI. Subsequently, fluorescence was recorded microscopically (Zeiss Axio Observer.Z1), and quantitatively at 380 nm (ApoBrite) and 617 nm (PI) using a plate reader (Tecan M200 Pro).

**Fluorescence microscopy**

Intestinal organoids were stained with nuclear dyes as described above and subsequently analyzed in hydrogel (Matrigel/BME) on a Zeiss Axio Observer.Z1 microscope. Brightfield images were taken with Palm-ROBO and fluorescence pictures with AxioVision Software (Zeiss).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism (GraphPad Prism Software, Inc.). Unless denoted otherwise, experiments were repeated three times with technical triplicates. One-way ANOVA with Dunnett’s multiple comparisons test was performed.

**RESULTS & DISCUSSION**

Cell death analysis in intestinal organoids in general, and its detection by PI/Hoechst staining in particular, requires precise experimental timing. If organoids are grown for prolonged periods of time dead cells accumulate in the lumen leading to a strong PI background. Therefore, all experiments shown were performed at day 3 after crypt isolation, whereas thawed human organoids were analyzed at day 3 after splitting. Moreover, cell death induction was performed overnight to ensure proper cell membrane disintegration and nuclear staining, independent of the mode of cell death. Initially, organoids were stained for various amounts of time (5–60 min) to assess the optimal duration of staining with...
both dyes. As stainings with both dyes were close to completion at 30 min, this time point was used for all subsequent experiments (Supplementary Figure 1).

Figure 1A shows exemplified pictures of murine intestinal organoids treated with the chemotherapeutic drug cisplatin or the pan-kinase inhibitor STS as a positive control. Whereas the PI signal gradually increased with increasing cisplatin concentrations, the Hoechst signal was only slightly attenuated (Figure 1A & B). Quantification of PI and Hoechst fluorescence enabled ratio formation, thus normalizing dying/dead organoids (PI-positive) to total DNA (Hoechst-positive). This ratio is significantly different between untreated organoids and increasing cisplatin concentrations, or STS (Figure 1C). The internal normalization by Hoechst staining stabilized measurements over a wide range of organoid densities (Figure 2B, right), whereas other established methods assessing cellular respiratory potential revealed a strong dependency on cell numbers (Figure 2A & B).

Having established that the ratio of PI/Hoechst signal gradually increased with increasing cell death induced by increasing cisplatin concentrations (Figure 1B), we next aimed at assessing treatment-specific organoid cell death in response to the chemotherapeutic drugs cisplatin and 5-fluorouracil (5-FU) (Figure 3A & B). Increasing concentrations of either chemotherapeutic drug resulted in an increased PI/Hoechst ratio, and an increase in the calculated treatment-specific organoid death (Figure 3A & B). To assess whether cell death observed was associated with apoptosis, caspase activity was analyzed in parallel with PI staining, demonstrating double-positive cells (Figure 3C) and a dose-dependent increase in caspase activity (Figure 3D). Currently, organoid cell death is frequently assessed indirectly by a reduction in intracellular ATP levels [28,29]. The direct comparison revealed that PI/Hoechst staining detects chemotherapy-induced organoid cell death as sensitively as intracellular ATP levels (Figure 3B & E).

In order to verify that cell death analysis by PI/Hoechst staining is not limited to murine primary intestinal organoids, we also assessed cell death quantification with PI/Hoechst staining in murine tumoroids (Figure 4A–C) and human intestinal organoids (Figure 4D), confirming the suitability of this method for other types of organoids.

We next set to multiplex cell death assessment by combining PI/Hoechst staining with our previously established method of measuring organoid viability by MTT reduction [30]. Therefore, organoids from intestinal tumors of APC<sup>min</sup> mice (tumoroids) were treated with indicated concentrations of 5-FU, stained with PI and Hoechst, and fluorescence was measured. Subsequently, PI/Hoechst-stained tumoroids were further incubated with MTT and reduction capacity was analyzed by absorbance of the resulting formazan at 562 nm. Whereas viable (ut) tumoroids efficiently reduced MTT to purple formazan (Figure 4A), tumoroids treated with 5-FU showed morphological disintegration and failed to reduce MTT (Figure 4B). A decrease in MTT reduction was paralleled by an increase in PI signal, but stable Hoechst staining (Figure 4A & B). Quantification of PI/Hoechst fluorescence and formazan absorption enabled calculation of treatment-specific organoid cell death (PI/Hoechst), which inversely correlated with decreased MTT reduction (Figure 4C). Multiplexing PI/Hoechst staining and MTT reduction was also confirmed by analyzing cisplatin-induced cell death in human
FINANCIAL & COMPETING INTERESTS DISCLOSURE

KJB was supported by a fellowship from the Baden-Württemberg Ministry of Science, Research and Art-funded Co-operative research training school ‘Advanced in vitro test systems for the analysis of cell-chemical interactions in drug discovery and environmental safety’ (InViTe). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

ETHICAL CONDUCT OF RESEARCH

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Papers of special note have been highlighted as ‘of interest’.


2. Murine intestinal organoids are described for the first time.


9. Murine and human intestinal organoids are investigated for the interplay of TNF and NF-κB in the intestine.


23. Patient-derived organoids are used as preclinical models.


28. The authors use the nuclear dye Hoechst and propidium iodide to microscopically assess organoid cell death.


33. Organoids are employed for a drug sensitivity assay us- ing a commercial kit detecting changes in intracellular ATP.


35. The authors describe how MTT reduction in intestinal organoids can be used to assess cell death.

Vol. 67 No. 1 © 2019 Thomas Brunner