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

In 1 collection

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BioTechniques

Organoid and Assembloid

Francesca Lake
 BioTechniques

ABSTRACT

This protocol describes 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 Hoechst 33342. 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 this method with established assays measuring mitochondrial activity further enhances its applicability in personalized medicine and drug discovery.

ATTACHMENTS

A fast and simple
 fluorometric method to
 detect cell death in 3D
 intestinal organoids-
 converted.pdf

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We use this protocol and it's working

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28661

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GUIDELINES

Troubleshooting

- Ensure organoid and Matrigel/BME integrity before starting treatment.
- Determine optimal time-point for start and endpoint of treatment (kinetics).
- Always add growth and staining medium gently to not disrupt Matrigel/BME domes.
- Check PI& Hoechst fluorescence microscopically before starting quantification.
- If effects of treatment are low, consider using thawed organoids rather than organoids derived from freshly isolated crypts, in order to reduce unspecific background (dead cells and cellular debris).

References

CITATION

Sato, T., Vries, R., Snippert, H. et al. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 549 (7244): 262-265.

LINK

<https://doi.org/10.1038/nature07935>

CITATION

Thomas GrabingerEugenia DelgadoThomas Brunner (2016). Analysis of Cell Death Induction in Intestinal Organoids In Vitro. *Methods in Molecular Biology* 1419: 83-93.

CITATION

Grabinger T, Luks L, Kostadinova F, Zimmerlin C, Medema JP, Leist M, Brunner T (2014). Ex vivo culture of intestinal crypt organoids as a model system for assessing cell death induction in intestinal epithelial cells and enteropathy. *Cell Death Dis* 5: e1228.

LINK

[10.1038/cddis.2014.183](https://doi.org/10.1038/cddis.2014.183)

MATERIALS

MATERIALS

- ☒ B-27 Supplement **Gibco - Thermo Fischer Catalog #17504044**
- ☒ DMEM/F12 w/o L-Glutamine HEPES 500 ml **Sigma Aldrich Catalog #D6421-500ML**
- ☒ N-2 Supplement (100X) **Thermo Fisher Catalog #17502048**
- ☒ Hoechst 33342, Trihydrochloride, Trihydrate, 100 mg **Thermo Fisher Catalog #H1399**
- ☒ Propidium Iodide - 1.0 mg/mL Solution in Water **Thermo Fisher Catalog #P3566**
- ☒ cis-Diammineplatinum(II) dichloride **Sigma Aldrich Catalog #P4394**
- ☒ 5-Fluorouracil **Sigma Aldrich Catalog #F6627**
- ☒ Staurosporine **Enzo Life Sciences Catalog #ALX-380-014-C250**
- ☒ Recombinant Murine Epidermal Growth Factor (mEGF) **peprotech Catalog #315-09**
- ☒ Noggin (mNoggin) **peprotech Catalog #250-38**
- ☒ Recombinant Human R-Spondin-1 (HR-Spondin 1) **peprotech Catalog #120-38**
- ☒ Cultrex® Reduced Growth Factor Basement Membrane Matrix Type 2 (BME 2) **Trevigen Catalog #3533-010-02**
- ☒ Dulbecco's Modified Eagle's Medium - high glucose **Sigma Aldrich Catalog #D1145**
- ☒ Penicillin-Streptomycin **Sigma Aldrich Catalog #P4333**
- ☒ Gentamicin solution **Sigma Aldrich Catalog #G1272**
- ☒ L-Glutamine solution **Sigma Aldrich Catalog #G7513**
- ☒ Dulbecco's Phosphate Buffered Saline **Sigma Aldrich Catalog #D8537**
- ☒ N-Acetyl-L-cysteine **Sigma Aldrich Catalog #A9165**
- ☒ Thiazolyl Blue Tetrazolium Bromide **Sigma Aldrich Catalog #M2128**
- ☒ HEPES **Carl Roth Catalog #9105.3**
- ☒ Albumin Fraction V protease-free (BSA) **Carl Roth Catalog #T844.3**
- ☒ 5ml pipet **Sarstedt Catalog #86.1253.001**
- ☒ 10ml serological pipette **Sarstedt Catalog #86.1254.001**
- ☒ 25ml serological pipette **Sarstedt Catalog #86.1685.001**
- ☒ 200ul pipette tip **Sarstedt Catalog #70.760.002**
- ☒ 10µl pipette tip neutral **Sarstedt Catalog #70.1130**
- ☒ Filter tip 1000uL **Sarstedt Catalog #70.762.411**
- ☒ Tube 15ml 120x17mm PP **Sarstedt Catalog #62.554.502**
- ☒ Tube 50ml 114x28mm PP **Sarstedt Catalog # 62.547.254**
- ☒ TC Plate 24 WellStandardF **Sarstedt Catalog #83.3922**
- ☒ TC Plate 96 WellStandardF **Sarstedt Catalog #83.3924**

Materials

Cell culture ware	Abbreviation/ Synonym	Catalog Number	Manufacturer
5ml pipet		861.253.001	Sarstedt
10ml pipet		861.254.001	Sarstedt
25ml pipet		861.685.001	Sarstedt
10 µl pipet tip		70.760.002	Sarstedt
200 µl pipet tip		70.1130	Sarstedt
1000 µl pipet tip		70.762.411	Sarstedt
15ml Falcon Tube	15ml Falcon	62.554.502	Sarstedt
50ml Falcon Tube	50ml Falcon	62.547.004	Sarstedt
24-well plate	24-well plate	83.3922	Sarstedt
96-well plate	96-well plate	83.3924	Sarstedt

Murine intestinal organoid growth medium

Supplement Advanced DMEM/F12 with 1mM 0.1 Mass Percent BSA , 1mM 2 Mass Percent L-glutamine , 1mM 10 Mass Percent HEPES , 1mM 100 U/ml penicillin , 1mM 100 µg/ml streptomycin , 1mM 1 Mass Percent N-acetyl cysteine (Sigma) , 1mM 1 x B27 supplement , 1mM 1 x N2 supplement (Gibco) , 1mM 50 ng/ml mEGF , and 1mM 1 ng/ml mNoggin (Peprotech) . Add hR-spondin-1 as conditioned medium of hR-spondin-1-transfected HEK 293T cells to a final volume of 25% (v/v).

Propidium Iodide and Hoechst33342 staining medium

Use fully supplemented medium (as described above) and add Propidium Iodide and Hoechst33342 up to a final concentration of 1mM 10 µg/ml PI& Hoechst . Add 80 µL of staining medium per well (96-well plate).

Equipment

Equipment	
Tecan Infinite M200 Pro	NAME
Platereader	TYPE
Tecan	BRAND
-	SKU

Equipment	
Axio Observer.Z1	NAME
Fluorescence Microscope	TYPE
Zeiss	BRAND
-	SKU

Equipment	
HeraCell CO2/O2-Incubator	NAME
Incubator	TYPE
Fisher Scientific	BRAND
-	SKU

Equipment

HeraSafe Laminar Flow

NAME

Fisher Scientific

BRAND

-

SKU

SAFETY WARNINGS

Please refer to the Safety Data Sheets associated with each reagent for safety information.

BEFORE START INSTRUCTIONS

Prepare all media and reagents required.

Ensure organoid and Matrigel or BME integrity before starting treatment.

Determine optimal time-point for start and endpoint of treatment (kinetics).

Check Propidium Iodide & Hoechst 33342 fluorescence microscopically before starting quantification.

Preparing organoids for staining

1

Grow murine intestinal organoids as described, preferably in clear 96-well plates.

CITATION

Sato, T., R. G. Vries, H. J. Snippert, M. van de Wetering, N. Barker, D. E. Stange, J. H. van Es, A. Abo, P. Kujala, P. J. Peters and H. Clevers (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459(7244): 262-265.

LINK

[10.1038/nature07935](https://doi.org/10.1038/nature07935)

2

Plate 200-300 crypts per well in 8 μ L Matrigel or BME, incubate for 00:20:00 at

37 °C 5% CO₂, and cover crypts dropwise with 80 μ L pre-warmed complete murine intestinal organoid growth medium (described in [Materials section](#)).

Note

Depending on whether starting with freshly isolated crypts or thawed organoids, determine timepoint/duration of treatment and analysis.

- 3 Allow cultures to grow for 3 days at 37°C 5% CO_2 before starting cell death treatment.

Staining of organoids with Propidium Iodide & Hoechst 333...

4

Note

Inspect organoid growth and integrity microscopically before starting treatment.

Treat organoids with Staurosporine or other substance for at least 16:00:00 to ensure disruption of plasma membranes.

Note

Use technical triplicates at minimum and include negative (untreated) and positive (with Staurosporine) controls.

- 5 Check effects of treatment — morphological differences in treated vs. untreated organoids.

- 6 Remove treatment medium and replenish organoids with pre-warmed staining medium with final concentration 10 Mass Percent Propidium Iodide & Hoechst 33342.

- 7 Stain for 00:30:00 at 37°C 5% CO_2 .

8 Replace staining medium with 80 µL pre-warmed, fresh phenol red-free medium.

9 Check fluorescence with microscope.

Quantification of Propidium Iodide & Hoechst 33342 fluores..

10 Start plate reader and measurement software (Tecan).

Software

Tecan

NAME

11 Remove the lid of the plate and set up the plate reader with the following sub-steps.

Note

Measurement can be performed at Room temperature, as it is an endpoint assay.

11.1 Set measurement from top position.

11.2 Gain must be set using the wells with the highest cell death (Propidium Iodide) and the lowest cell death (Hoechst 33342). The values should fall between 115 and 160 (positive and negative control).

If measurement values do not fall within the range, readjust gain and remeasure.

11.3

Allow the plate reader to determine the optimal Z-position automatically from the corresponding wells. Check for values between 1.5×10^6 and $1.6 \times 10^6 \mu\text{m}$. (This can take up to 00:02:00 .)

12

Measure all wells for Propidium Iodide fluorescence and wait 00:00:30 before measuring Hoechst 33342 fluorescence.

Measure fluorescence of each well with 25 flashes and an integration time of 20 μs . Lag and settle time should be set to 0 seconds. Use 4x4 measurements per well, with a border of 1mm around measurement points (measurement points can be increased to counter uneven organoid distribution at the cost of measurement speed).

Note

Excitation and emission wavelengths for PI are 535 nm and 617 nm, respectively. For Hoechst, 361 nm and 486 nm should be used as excitation and emission wavelengths.

13

Calculate Propidium Iodide/Hoechst 33342 (PI/Hoechst) ratio using relative fluorescence units.

$$\frac{\text{PI}}{\text{H}} \text{ratio} = \frac{\text{RFU(PI)}}{\text{RFU(Hoechst)}}$$

14

Use PI/Hoechst ratios to quantify treatment-specific organoid cell death by dividing each sample with the positive control (e.g. containing Staurosporine), multiplying the value by 100, and then subtracting the PI/H ratio of the untreated samples.

$$\text{treatment\; specific\; organoid\; cell\; death\; [\%]} = \frac{x(\text{sample})}{z(\text{STS})} \times 100 - y(\text{ut})$$