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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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1 Works for me dx.doi.org/10.17504/protocols.io.78vhrw6

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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.

EXTERNAL LINK

https://www.future-science.com/doi/10.2144/btn-2019-0023

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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

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

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

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

Grabinger T, Luks L, Kostadinova F, Zimberlin 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. http://10.1038/cddis.2014.183

MATERIALS

NAME	CATALOG #	VENDOR
B-27 Supplement	17504044	Gibco - Thermo Fischer
DMEM/F12 w/o L-Glutamine HEPES 500 ml	D6421-500ML	Sigma Aldrich
N-2 Supplement (100X)	17502048	Thermo Fisher
Hoechst 33342, Trihydrochloride, Trihydrate, 100 mg	H1399	Thermo Fisher
Propidium Iodide - 1.0 mg/mL Solution in Water	P3566	Thermo Fisher
cis-Diammineplatinum(II) dichloride	P4394	Sigma Aldrich
5-Fluorouracil	F6627	Sigma Aldrich
Staurosporine	ALX-380-014-C250	Enzo Life Sciences
Recombinant Murine Epidermal Growth Factor (mEGF)	315-09	peprotech
Noggin (mNoggin)	250-38	peprotech
Recombinant Human R-Spondin-1 (HR-Spondin 1)	120-38	peprotech
Cultrex® Reduced Growth Factor Basement Membrane Matrix Type 2 (BME 2)	3533-010-02	Trevigen
Dulbecco's Modified Eagle's Medium - high glucose	D1145	Sigma Aldrich
Penicillin-Streptomycin	P4333	Sigma Aldrich
Gentamicin solution	G1272	Sigma Aldrich
L-Glutamine solution	G7513	Sigma Aldrich
Dulbecco's Phosphate Buffered Saline	D8537	Sigma Aldrich
N-Acetyl-L-cysteine	A9165	Sigma Aldrich
Thiazolyl Blue Tetrazolium Bromide	M2128	Sigma Aldrich
HEPES	9105.3	Carl Roth
Albumin Fraction V protease-free (BSA)	T844.3	Carl Roth

NAME	CATALOG #	VENDOR
5ml pipet	86.1253.001	Sarstedt
10ml serological pipette	86.1254.001	Sarstedt
25ml serological pipette	86.1685.001	Sarstedt
200ul pipette tip	70.760.002	Sarstedt
10µl pipette tip neutral	70.1130	Sarstedt
Filter tip 1000uL	70.762.411	Sarstedt
Tube 15ml 120x17mm PP	62.554.502	Sarstedt
Tube 50ml 114x28mm PP	62.547.254	Sarstedt
TC Plate 24 WellStandardF	83.3922	Sarstedt
TC Plate 96 WellStandardF	83.3924	Sarstedt
Corning® Matrigel® Basement Membrane Matrix growth factor reduced (GFR)	734-0269	Vwr

MATERIALS TEXT

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 [M]**0.1 undefined BSA** , [M]**2 undefined L-glutamine** , [M]**10 undefined HEPES** , [M]**100 U/ml penicillin** , [M]**100 µg/ml streptomycin** , [M]**1 undefined N-acetyl cysteine (Sigma)** , [M]**1 x B27 supplement** , [M]**1 x N2 supplement (Gibco)** , [M]**50 ng/ml mEGF** , and [M]**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 [M]**10 µg/ml PI& Hoechst** . Add **80 µl** of staining medium per well (96-well plate).

Equipment

Tecan Infinite M200 Pro
Platereader
Tecan -

Axio Observer.Z1
Fluorescence Microscope
Zeiss -

HeraCell CO2/O2-Incubator
Incubator
Fisher Scientific -

HeraSafe Laminar Flow
Fisher Scientific -

SAFETY WARNINGS

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

BEFORE STARTING

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.

> Sato, T., R. G. Vries, H. J. Snippert, M. van de Wetering, N. Barker, D. E. Stange, J. H. van Es, A. Abo, P. J. 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. http://10.1038/nature07935

2

Plate 200-300 crypts per well in **8 µl** Matrigel or BME, incubate for **00:20:00** at **37 °C** **5% CO2** , and cover crypts dropwise with **80 µl** **pre-warmed** complete murine intestinal organoid growth medium (described in Materials section).

> 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% CO2** before starting cell death treatment.

Staining of organoids with Propidium Iodide & Hoechst 33342

4

> 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.

> 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 Mg/ml** Propidium Iodide & Hoechst 33342.

7 Stain for 00:30:00 at 37 °C 5% CO2 .

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 fluorescence

10 Start plate reader and measurement software (Tecan).

Tecan

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

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 $1.5x10^6$ and $1.6x10^6$ µm. (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).

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.

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

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.

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