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

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We use this protocol and it's working

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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.

Attachments

A fast and simple fl...

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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.

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

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, 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.

10.1038/cddis.2014.183 LINK

Materials

MATERIALS

- B-27 Supplement **Gibco - Thermo Fisher Scientific** **Catalog #**17504044
- DMEM/F12 w/o L-Glutamine HEPES 500 ml **Merck MilliporeSigma (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 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P4394
- 5-Fluorouracil **Merck MilliporeSigma (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 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D1145
- Penicillin-Streptomycin **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P4333
- Gentamicin solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G1272
- L-Glutamine solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G7513
- Dulbecco's Phosphate Buffered Saline **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D8537
- N-Acetyl-L-cysteine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A9165
- Thiazolyl Blue Tetrazolium Bromide **Merck MilliporeSigma (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 120×17mm PP **Sarstedt** **Catalog #**62.554.502

Tube 50ml 114×28mm PP **Sarstedt Catalog #** 62.547.254

TC Plate 24 WellStandardF **Sarstedt Catalog #**83.3922

TC Plate 96 WellStandardF **Sarstedt Catalog #**83.3924

Corning® Matrigel® Basement Membrane Matrix growth factor reduced (GFR) **VWR International (Avantor) Catalog #**734-0269

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 Mass Percent BSA , [M] 2 Mass Percent L-glutamine , [M] 10 Mass Percent HEPES , [M] 100 U/ml penicillin , [M] 100 µg/ml streptomycin , [M] 1 Mass Percent 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

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

NAME	BRAND	SKU
HeraSafe Laminar Flow	Fisher Scientific	-

Safety warnings

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

Before start

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.

10.1038/nature07935 LINK

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**).

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% CO2 before starting cell death treatment.

Staining of organoids with Propidium Iodide & Hoechst 33342

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 [M] 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).

Software

NAME	SOURCE LINK
Tecan	https://lifesciences.tecan.com

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 1.6×10^6 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 4×4 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.

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

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.

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

Citations

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Step 1

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. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche
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