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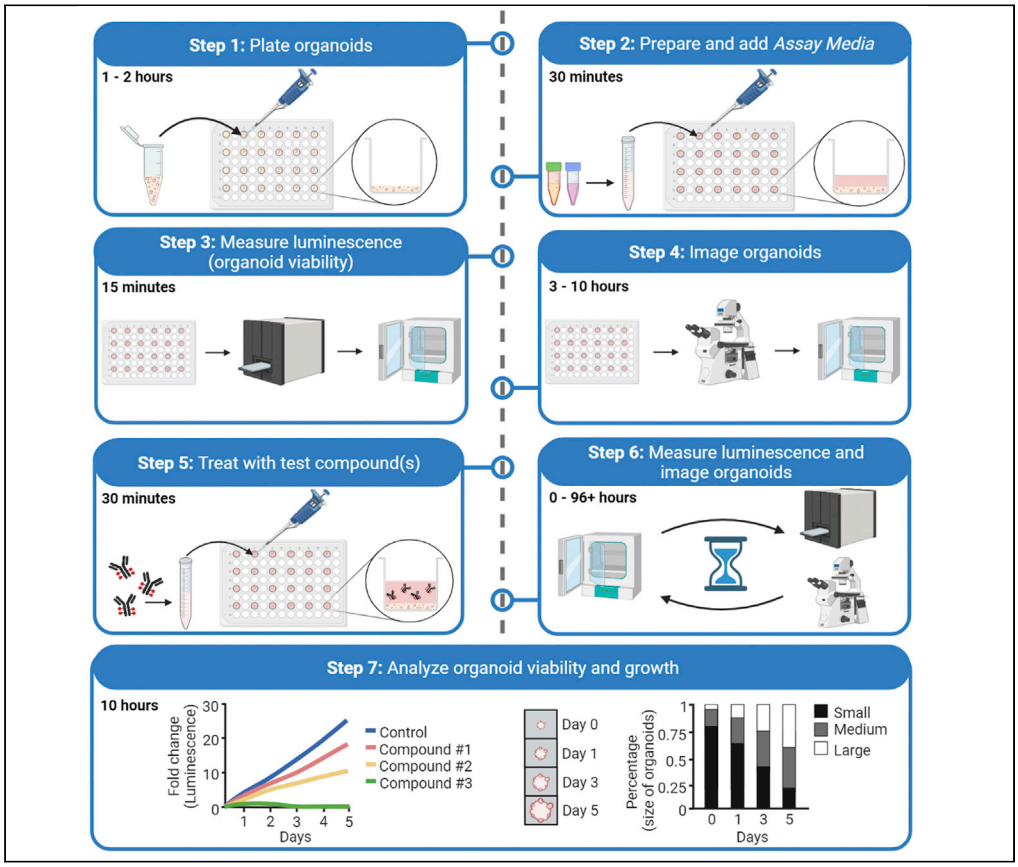
Protocol

Longitudinal quantification of mouse gastric tumor organoid viability and growth using luminescence and microscopy



Tumor-derived organoids are valuable for testing anti-cancer drugs *in vitro*, but existing lysis-based protocols for viability measurement are laborious and restricted at a single time point. Here, we provide a lysis-free protocol for longitudinal and rapid assessment of mouse gastric tumor organoid viability and growth. We describe organoid plating, viability assessment via luminescence measurement, quantification of organoid growth by microscopy imaging, and treatment of organoids with test compounds to evaluate the effects on viability and growth at various time points.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Longitudinal quantification of mouse tumor organoid viability and growth

Uses luminescence and microscopy and does not require organoid lysis

Can be used to evaluate the response of tumor organoids to anti-cancer drugs

Applicable to organoids derived from other mouse or human tumors and healthy tissues

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Longitudinal quantification of mouse gastric tumor organoid viability and growth using luminescence and microscopy

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SUMMARY

Tumor-derived organoids are valuable for testing anti-cancer drugs *in vitro*, but existing lysis-based protocols for viability measurement are laborious and restricted at a single time point. Here, we provide a lysis-free protocol for longitudinal and rapid assessment of mouse gastric tumor organoid viability and growth. We describe organoid plating, viability assessment via luminescence measurement, quantification of organoid growth by microscopy imaging, and treatment of organoids with test compounds to evaluate the effects on viability and growth at various time points.

BEFORE YOU BEGIN

This protocol utilizes organoids derived from gastric tumors of 14-week-old *Gp130*^{Y757F} mice[1] of either gender, which were generated and maintained as previously described.[2] However, this protocol can also be applied to organoids generated from other mouse tumor models, human tumors, and healthy tissues. Users are encouraged to optimize this protocol to accommodate for differences in culture conditions (e.g., addition of supplementary reagents, such as hormones), as well as the growth kinetics of their selected organoids. This includes determining the optimal density for plated organoids to ensure robust growth.

Institutional permissions

All animal studies were approved and conducted in accordance with the Animal Ethics Committee at La Trobe University and the Austin Health precinct. Users are reminded to seek appropriate permissions from their relevant institutions to conduct studies involving animal or human tissues.

Passage and maintenance of organoids

Timing: 1 h

1. Passage organoids every 5–7 days in *Basement Membrane Matrix* (Cultrex® Reduced Growth Factor BME, Type 2 PathClear®; R&D Systems).
   a. Refer to steps 1–14 of the '*Plate organoids for assay*' section for detailed instructions on how to passage organoids.

Note: In this protocol, 6 wells of a 24-well tissue culture plate each containing 40 organoids in 50 μL of *Basement Membrane Matrix* were grown for 5–7 days to yield sufficient material

(approximately 1200 organoids) for a single assay plate (i.e., 21 wells of a 96-well plate, each well containing 35 organoids).

2. Supplement each organoid dome with 500 µL of *Organoid Growth Media*.

Reagent	Final concentration	Volume
IntestiCult™ OGM Mouse Supplement 1	1×	5 mL
IntestiCult™ OGM Mouse Supplement 2	1×	5 mL
IntestiCult™ OGM Mouse Basal Media	1×	90 mL
Total	–	**100 mL**

Note: *Organoid Growth Media* can be stored as 10 mL aliquots at −20°C. Once thawed at room temperature (approximately 20°C–25°C), aliquots can be stored at 4°C for up to 14 days.

3. Replenish wells with 500 µL of *Organoid Growth Media* every 2–3 days.

Note: Cultures in which more than 40% of organoids are >200 µm in diameter or have reached approximately 70% confluence should be passaged to avoid necrosis.[3] Necrotic organoids will cease to proliferate and are easily distinguishable by their dark lumen.[4–6]

Note: For a single assay plate, we recommend that a minimum of 30 and a maximum of 40 organoids are plated per well. However, users may wish to optimize this density depending on the growth kinetics of their selected organoids.

Preparation prior to commencing protocol

Timing: 1–16 h

4. Prior to passaging organoids and commencing the protocol, thaw *Basement Membrane Matrix* on ice.
   a. At least three biological replicates (each comprising technical triplicates) should be used for each experimental condition.
   b. An example for calculating the amount of *Basement Membrane Matrix* required for a single assay plate (i.e., treatment of one biological replicate with a single compound at 6 different concentrations) is shown below:
      i. *Number of wells for a single assay plate* = (6 *concentrations of a test compound* × 3 *technical replicates for each concentration*) + 3 *blank wells* + 3 *control wells* + 10 *extra wells* = 34 *wells*
      ii. *Basement Membrane Matrix required for a single assay plate* = 34 *wells* × 35µ*L per well* = 1190 µ*L*

Note: Once thawed, Basement Membrane Matrix can be stored as 600 µL aliquots at −80°C. Avoid freeze-thawing Basement Membrane Matrix aliquots more than once.

Note: Users are encouraged to calculate extra volumes of *Basement Membrane Matrix* to compensate for plating inaccuracy or dead volume required for dispensing. We recommend calculating enough to accommodate 10 extra wells of *Basement Membrane Matrix* as shown in the equation above.

5. Pre-cool white, opaque-walled, clear-bottomed 96-well tissue culture plates on ice at 4°C. This will facilitate even dispersion of the *Basement Membrane Matrix* when plating the assay.

6. Prepare 10 mL of fresh *Organoid Growth Media* and warm to room temperature (approximately 20°C–25°C).
7. Prepare *Wash Media*.

Reagent	Final concentration	Volume
Heat-inactivated FBS	1×	50 mL
Advanced DMEM/F-12	1×	445 mL
Pen-Strep	1×	5 mL
Total	–	**500 mL**

Note: *Wash Media* can be stored at 4°C for up to 4 weeks. *Wash Media* should be warmed to room temperature (approximately 20°C–25°C) prior to commencing protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Cultrex® Reduced Growth Factor BME, Type 2 PathClear®	R&D Systems	Cat#3533-010-02
Gentle Cell Dissociation Reagent	STEMCELL Technologies	Cat#100-0485
IntestiCult™ Organoid Growth Media (Mouse)	STEMCELL Technologies	Cat#06005
Advanced DMEM/F-12	Gibco	Cat#12634010
Penicillin-Streptomycin (Pen-Strep) (10,000 U/mL)	Gibco	Cat#15140122
Heat-inactivated fetal bovine serum (FBS)	Bovogen Biologicals	Cat#SFBS
Critical commercial assays		
RealTime-Glo™ MT Cell Viability Assay	Promega	Cat#G9713
Experimental models: Cell lines		
Organoids derived from gastric tumors of *Gp130*^{Y757F} mice (recommended passage range: 5–10)	In-house	MGI:2388478
Software and algorithms		
Prism version 9.3.1	GraphPad	https://www.graphpad.com/scientific-software/prism/
Kaleido™ data acquisition & analysis software	PerkinElmer	Implemented in Cat#HH34000000
OlyVIA image software	Olympus	https://www.olympus-lifescience.com/en/discovery/image-sharing-made-easy-meet-olyvia/
Other		
Cell culture microplate, 96-well, ps, f-bottom (chimney well), µclear®, white, Cellstar®, lid with condensation rings	Greiner Bio-One	Cat#655098
24-well clear flat bottom TC-treated multi-well cell culture plate with lid, sterile	Falcon	Cat#353047
EnSight® multimode plate reader	PerkinElmer	Cat#HH34000000
37°C, 5–10% CO_2 incubator	N/A	N/A
Orbital roller	N/A	N/A
1.5 mL microcentrifuge tube	Eppendorf	Cat#0030123328
15 mL polypropylene conical centrifuge tube	Greiner Bio-One	Cat#188261
Swinging bucket rotor centrifuge	N/A	N/A
Benchtop mini/micro fixed angle rotor centrifuge	N/A	N/A
Class II biological safety cabinet	N/A	N/A
Inverted microscope	Olympus	IX81

MATERIALS AND EQUIPMENT

Alternatives: This protocol uses Cultrex® Reduced Growth Factor BME, Type 2 PathClear® as the *Basement Membrane Matrix* to support the growth and maintenance of mouse gastrointestinal tumor organoids. This *Basement Membrane Matrix* is expected to be compatible with organoids derived from other tissue types; however, users should optimize growth conditions according to their specific needs. Users should also plate blank wells that only contain *Basement Membrane Matrix* and *Organoid Growth Media* (i.e., no organoids) to assess background levels of luminescence produced by their selected *Basement Membrane Matrix* and *Organoid Growth Media*.

Alternatives: This protocol utilizes IntestiCult™ Organoid Growth Media (Mouse) for the growth of organoids derived from mouse gastrointestinal tumors. Users are expected to trial their preferred *Organoid Growth Media* to confirm its compatibility with this protocol. Different components of *Organoid Growth Media* may affect enzymatic performance and luminescence signal of the RealTime-Glo™ MT Cell Viability Assay.

Alternatives: This protocol utilizes organoids derived from gastric tumors of *Gp130*^{Y757F} mice.[1] However, we have also successfully utilised organoids derived from colon tumors of *Cdx2*^{CreERT2};*Apc*$^{fl/fl}$ mice.[7] While we anticipate that this workflow will have broad applicability for different organoids, users are encouraged to determine the optimal density of their selected organoids to ensure robust growth. Users may also need to supplement their *Organoid Growth Media* with additional reagents (e.g., hormones) depending on the specific growth requirements of their selected organoids.

Alternatives: This protocol uses white, opaque-walled, clear-bottomed 96-well tissue culture plates to maximise signal intensity and reduce background interference between adjacent wells. These plates also allow for microscopy and comparative analysis of organoid diameter as a surrogate measure of growth. Black, opaque-walled, clear-bottomed 96-well tissue culture plates can also be used, but may reduce luminescence due to quenching.

Alternatives: This protocol uses an EnSight® Multimode Plate Reader (PerkinElmer) with Kaleido™ data acquisition & analysis software (PerkinElmer). An EnSight® Multimode Plate Reader enables detection of luminescence from a small number of organoids per well. This provides a cost-effective solution and overcomes limitations of securing large amounts of starting material. The EnSight® Multimode Plate Reader also allows users to maintain their organoids at 37°C while measuring luminescence, which is critical to the performance of the enzymatic activity of the RealTime-Glo™ MT Cell Viability Assay. If an EnSight® Multimode Plate Reader is unavailable, an alternative luminometer capable of holding a 96-well tissue culture plate may be used. However, other luminometers may not be able to detect luminescence to the same sensitivity as the EnSight® Multimode Plate Reader.

Alternatives: The intensity of luminescence produced by organoids depends on several environmental factors, including temperature. If the selected luminometer does not have temperature control, users should measure luminescence immediately after removing their assay from the 37°C incubator to prevent cooling. Users should also check which direction (bottom or top) their selected luminometer measures luminescence, as this will determine whether clear or opaque bottomed 96-well tissue culture plates are used.

Alternatives: Other image analysis software (e.g., ImageJ) may also be used to manually draw lines for measuring organoid diameter (growth).

STEP-BY-STEP METHOD DETAILS

Plate organoids

Timing: 1–2 h

This step involves plating organoids in a white, opaque-walled, clear-bottomed 96-well tissue culture plate to measure organoid viability and growth. For this protocol, cryopreserved organoid fragments derived from gastric tumors of *Gp130*^{Y757F} mice were used.[1,2]

1. At time of passaging, manually count the number of organoids in each well of a 24-well tissue culture plate under a bright field microscope and record the total number of organoids observed.
2. Aspirate and discard *Organoid Growth Media*.
3. Add 1 mL of 4°C cold Gentle Cell Dissociation Reagent (STEMCELL Technologies) to each dome.
   a. Using a 1,000 μL pipette tip, mechanically disrupt each organoid *Basement Membrane Matrix* dome by gently pipetting Gentle Cell Dissociation Reagent onto each dome 10 times to break the organoids into smaller fragments.

⚠ CRITICAL: After disrupting each organoid *Basement Membrane Matrix* dome, pool the suspension into a 15 mL conical tube.

4. Rinse each well with 1 mL of 4°C cold Gentle Cell Dissociation Reagent and add this to the same 15 mL conical tube.
5. Place tube on an orbital roller at room temperature (approximately 20°C–25°C) for 10 min.
6. Centrifuge at 400 × *g* at 4°C for 5 min using a swinging bucket rotor centrifuge to maximize recovery of the organoid pellet.
7. Carefully aspirate and discard the supernatant without disturbing the organoid pellet.
8. Resuspend the organoid pellet in 5 mL of cold *Wash Media*.
9. Centrifuge at 400 × *g* at 4°C for 5 min.
10. Carefully aspirate and discard the supernatant without disrupting the organoid pellet.
11. Resuspend the organoid pellet in 1 mL of cold *Wash Media*.
12. Transfer the resuspended organoid pellet into a 1.5 mL microcentrifuge tube.
13. Centrifuge at 9,600 × *g* at 4°C for 5 min using a benchtop mini/micro fixed angle rotor centrifuge to maximize recovery of the organoid pellet.

Note: The organoids used in this protocol can survive high centrifugal forces. Users may need to optimize centrifugal forces to accommodate their selected organoids.

14. Carefully aspirate and discard the supernatant without disrupting the organoid pellet.
15. Gently resuspend the pellet in ice cold *Basement Membrane Matrix*.
    a. The volume required depends on the number of experimental conditions. At least three biological replicates (each containing technical triplicates) for each condition should be used.
    b. An example for calculating the amount of *Basement Membrane Matrix* required for a single assay plate (i.e., treatment of one biological replicate with a single compound at 6 different concentrations) is shown below:
       i. *Number of wells for a single assay plate* = (6 *concentrations of a test compound* × 3 *technical replicates for each concentration*) + 3 *blank wells* + 3 *control wells* + 10 *extra wells* = 34 *wells*
       ii. *Basement Membrane Matrix required for a single assay plate* = 34 *wells* × 35μL *per well* = 1190 μL

Note: For a single assay plate, 21 wells each containing 35 organoids resuspended in 35 μL of *Basement Membrane Matrix* was used. We recommend that a minimum of 30 and a maximum

Table 1. Example of an experimental layout in a 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	B		C		E1		E2		E3		E4	
B												
C	B		C		E1		E2		E3		E4	
D												
E	B		C		E1		E2		E3		E4	
F												
G	E5		E5		E5		E6		E6		E6	
H												

Each condition contains technical triplicates (i.e., three separate wells) with a gap between each well containing organoids to reduce interference of luminescence. B: Blank (wells containing only *Basement Membrane Matrix* and *Organoid Growth Media* (i.e., no organoids)). C: Control (untreated organoids). E#: Experimental Condition (organoids to be treated with a particular compound(s)).

of 40 organoids are plated per well. However, users may wish to optimize this density depending on the growth kinetics of their selected organoids to ensure robust growth.

Note: To ensure even distribution of organoids throughout the *Basement Membrane Matrix* mix, gently resuspend organoids by pipetting the mix after plating 5 wells.

⚠ CRITICAL: Do not introduce bubbles into the *Basement Membrane Matrix* mix when resuspending and plating organoids, as this will interfere with downstream imaging.

⚠ CRITICAL: Always maintain microcentrifuge tubes containing organoids resuspended in *Basement Membrane Matrix* on ice to prevent solidification.

16. Keep the white, opaque-walled, clear-bottomed 96-well tissue culture plate on ice in a Class II biological safety cabinet.

Note: Ensure the plate is level so that the organoid *Basement Membrane Matrix* mix will evenly coat the base of each well.

17. Dispense 35 µL of the organoid *Basement Membrane Matrix* mix directly into the center of each well.

Note: To reduce interference of luminescence between wells on the 96-well plate, leave a gap between each well containing organoids when plating (Table 1).

⚠ CRITICAL: Users should include three blank wells that only contain *Basement Membrane Matrix* and *Organoid Growth Media* (i.e., no organoids) to assess background levels of luminescence produced by their selected *Basement Membrane Matrix* and *Organoid Growth Media*.

18. Place the white, opaque-walled, clear-bottomed 96-well tissue culture plate into a 37°C incubator with 10% CO_2 for 15 min to solidify the organoid *Basement Membrane Matrix*.

Prepare and add *Assay Media*

Timing: 30 min

This step involves preparation of the *Assay Media* to measure organoid viability.

19. During the 15 min incubation period, prepare *Assay Media*.
    a. Equilibrate the RealTime-Glo™ MT Cell Viability Assay reagents NanoLuc® Enzyme (1000×) and MT Cell Viability Substrate (1000×) to 37°C by placing in an incubator for approximately 10 min or until thawed.
    b. Once thawed, prepare the *Assay Media* by adding the 1000× enzyme and 1000× substrate to *Organoid Growth Media* so the final concentration of each is 1×.
    c. 100 µL of *Assay Media* is required for each well.

⚠ CRITICAL: Prepare the *Assay Media* away from direct light to reduce degradation of the RealTime-Glo™ MT Cell Viability Substrate.

20. After the 15 min incubation period, add 100 µL of *Assay Media* to each well in a Class II biological safety cabinet.
21. Return the plated organoids to a 37°C, 10% CO_2 incubator for at least 16 h. 24 h is preferable to allow organoids to establish.

Measure luminescence (organoid viability)

Timing: 15 min

This step involves measuring luminescence signal as a direct correlation with organoid viability.

22. Following overnight (24 h) incubation of organoids in *Assay Media*, measure luminescence using a plate reading luminometer with a suitable integration time (0.25 s–1 s).

Note: In this protocol, an EnSight® Multimode Plate Reader with Kaleido™ data acquisition & analysis software was used. The EnSight® Multimode Plate Reader was maintained at 37°C and an integration time of 1 s was used.

⚠ CRITICAL: Protect the assay from direct light to reduce degradation of the RealTime-Glo™ MT Cell Viability Substrate.

⚠ CRITICAL: The assay should be maintained at, or as close to, 37°C when measuring luminescence. This is critical to the performance of the RealTime-Glo™ MT Cell Viability Assay. A luminometer with temperature control is recommended. If a luminometer with temperature control is unavailable, users should measure luminescence immediately after removing their assay from the 37°C incubator to prevent cooling.

⚠ CRITICAL: Luminometers that are in a Class II biological safety cabinet are recommended to maintain sterility.

23. After measuring luminescence, proceed to the next step (imaging).

Image organoids

Timing: 3–10 h

This step involves imaging organoids for quantification of diameter as a surrogate measure of organoid growth. This is important as it is difficult to determine whether a reduced luminescence signal observed from the RealTime-Glo™ MT Cell Viability Assay is due to organoid senescence or death.

24. Immediately after measuring luminescence, take photomicrographs of each well using an inverted microscope with suitable magnification, z-stack capability, and an on-stage incubator with temperature and CO_2 control.

Note: In this protocol, an IX81 Olympus inverted microscope with OlyVIA image software was used. The stage incubator of the IX81 Olympus inverted microscope was pre-heated to 37°C with 10% CO_2. The IX81 Olympus microscope is automated and motorized, allowing for specific stage positions to be saved. This allows for precise imaging of organoid wells at the same position for the duration of the assay.

Note: Users are required to determine the upper and lower z-stack limits and associated step sizes for their assay. In this protocol, a z-stack involving 10 steps spaced ~50 μm apart was used.

⚠ CRITICAL: Users should acquire multiple photos of each well per time point. The number of photos acquired will depend on the number of z-stack step sizes used.

⚠ CRITICAL: Protect the assay from direct light to reduce degradation of the RealTime-Glo™ MT Cell Viability Substrate.

25. Secure the assay plate to the microscope stage.
26. Take full-well photomicrographs of each organoid well with suitable upper and lower z-stack limits and associated step sizes.
27. After imaging is complete, return the assay to a 37°C, 10% CO_2 incubator.

Treat with test compound(s)

Timing: 30 min

This step involves the preparation and addition of test compound(s) to organoids. Viability and growth can be measured by luminescence and microscopy, respectively, at various time points.

28. Dilute desired concentrations of test compound(s) at 2× in *Assay Media*.
29. Overlay 100 μL of diluted test compound(s) to the pre-existing 100 μL per well at a 2× concentration (to make 1×).

Note: This approach allows users to avoid unnecessary aspiration of existing media, which may increase the risk of damaging organoids.

30. Return the assay to the incubator and remove only when measuring viability and imaging organoids at desired time points.

Note: We have previously used this protocol to measure organoid viability and growth over 11 consecutive days. However, users are required to optimize the maximum amount of time that their organoids can be maintained in culture for this assay.

Analysis

Timing: 10 h

This step involves quantification of organoid viability and growth by measuring luminescence and diameter, respectively.

Table 2. RLU and corrected RLU values of organoids at Day 0

Biological replicate B1			Biological replicate B2			Biological replicate B3		
n1	*n2*	*n3*	*n1*	*n2*	*n3*	*n1*	*n2*	*n3*
RLUs of blank wells (technical triplicates)			RLUs of blank wells (technical triplicates)			RLUs of blank wells (technical triplicates)		
436	424	458	435	517	485	431	486	466
RLUs of experimental condition (technical triplicates)			RLUs of experimental condition (technical triplicates)			RLUs of experimental condition (technical triplicates)		
7128	7830	7147	7140	9825	9178	8723	9332	8573
Corrected RLU			Corrected RLU			Corrected RLU		
6929			8235			8415		

Note: This protocol allows users to measure the viability of the same organoids over the course of treatment. This is an advantage over existing lysis-based methods,[8–11] where quantification of organoid viability can only be performed at a single experimental end-point.

Quantification of organoid viability by luminescence

The RealTime-Glo™ MT Cell Viability Assay measures the metabolic activity of viable organoids, which is provided as a luminescence signal. The amount of luminescence signal measured by a luminometer is expressed in units known as Relative Light Units (RLUs). Therefore, RLUs correlate with viability.

Note: In this protocol, RLUs of each well were collected using an EnSight® Multimode Plate Reader with Kaleido™ data acquisition & analysis software.

Example RLUs and calculation of fold-change in viability across two time points are shown in Tables 2–4 (3 biological replicates B1-B3, each comprising technical triplicates n1-n3).

31. Calculate corrected RLUs as follows:
    a. Calculate the average RLU (i.e., from technical triplicates) of blank wells and each experimental condition:

$$Average\ RLU = \frac{Technical\ triplicates\ n1 + n2 + n3}{3}$$

    b. Subtract the average RLU of blank wells from the average RLU of each experimental condition to determine the corrected RLU:

$$Corrected\ RLU = Average\ RLU\ of\ each\ experimental\ condition - average\ RLU\ of\ blank\ wells$$

32. Calculate fold change in viability across different time points by dividing the corrected RLU of each time point by the corrected RLU of the first time point.

Table 3. RLU and corrected RLU values of organoids at Day 5

Biological replicate B1			Biological replicate B2			Biological replicate B3		
n1	*n2*	*n3*	*n1*	*n2*	*n3*	*n1*	*n2*	*n3*
RLUs of blank wells (technical triplicates)			RLUs of blank wells (technical triplicates)			RLUs of blank wells (technical triplicates)		
684	826	890	772	806	811	821	752	845
RLUs of experimental condition (technical triplicates)			RLUs of experimental condition (technical triplicates)			RLUs of experimental condition (technical triplicates)		
22219	33000	25500	31453	40770	47689	31674	35986	35699
Corrected RLU			Corrected RLU			Corrected RLU		
26106			39174			33647		

Table 4. Fold-change in organoid viability at Day 0 vs Day 5

	Biological replicate B1	Biological replicate B2	Biological replicate B3
Day 0	1	1	1
Day 5	3.77	4.76	4.00

$$\textit{Fold change in viability} = \frac{\textit{Corrected RLU at selected timepoint}}{\textit{Corrected RLU at first timepoint}}$$

Quantification of organoid growth by microscopy

This protocol also combines the use of microscopy to longitudinally quantify organoid diameter as a surrogate measure of growth. Photomicrographs of each well were captured using an Olympus IX81 microscope (Figure 1).

> ***Note:*** Users will have acquired multiple photos of each well per time point. The number of photos acquired will depend on the number of z-stack step sizes used.

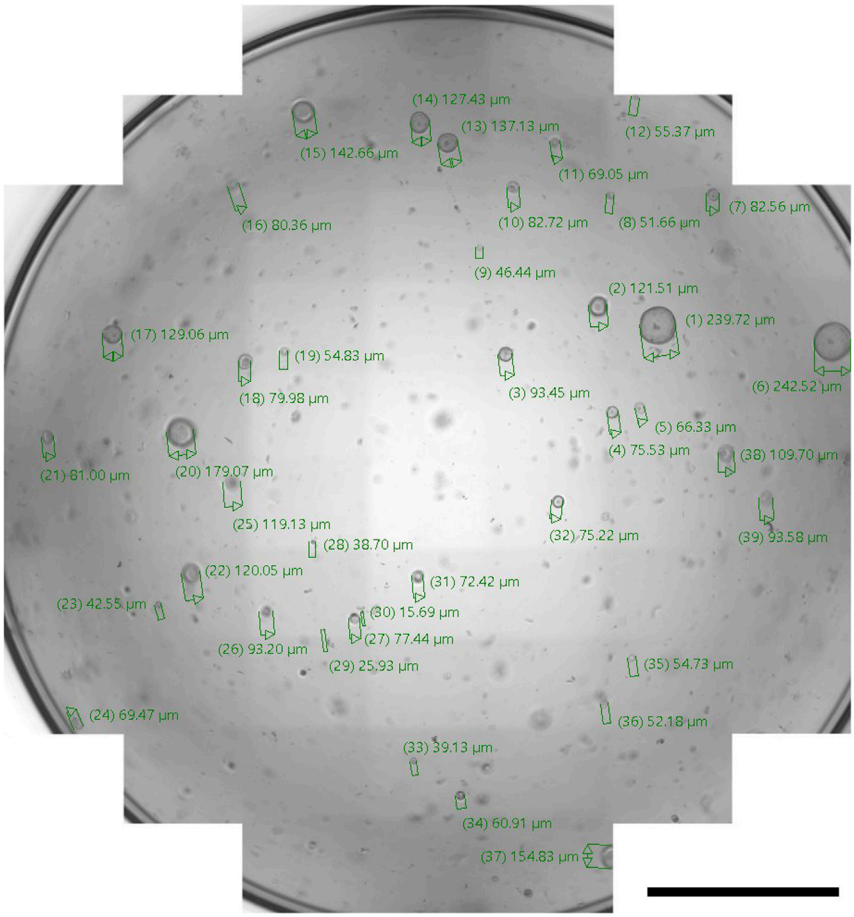

Figure 1. Representative single z-stack photomicrograph of an organoid well at Day 5

A line was drawn across each organoid at each time point to quantify growth. Number in brackets correspond to specific organoids. Only organoids that are in focus were measured in this image. Remaining organoids were quantified in other z-stack images where a clearer image was obtained. Measurements shown are the diameter of each organoid. Scale bar: 1 mm.

Table 5. Organoid diameters (μm) and corresponding sizes on Days 0 and 5

Organoid number	Organoid diameter Day 0 (μm)	Organoid size Day 0	Organoid diameter Day 5 (μm)	Organoid size Day 5
1	10 μm	Small	95 μm	Small
2	30 μm	Small	120 μm	Medium
3	50 μm	Small	220 μm	Large
4	220 μm	Large	485 μm	Large
5	80 μm	Small	210 μm	Large
6	60 μm	Small	150 μm	Medium
7	100 μm	Medium	320 μm	Large
8	150 μm	Medium	350 μm	Large
9	80 μm	Small	280 μm	Large
10	310 μm	Large	500 μm	Large

33. Calculate the diameter of each organoid by drawing a line spanning the width of an organoid using the *Measure* function in the OlyVIA image software.
34. Repeat until the diameter of every organoid is determined.

⚠ CRITICAL: Users should only measure organoids that are in focus (Figure 1) and avoid measuring the same organoid that may be captured in more than one image. Thus, users will need to identify the clearest image of each organoid in a z-stack for quantification.

Note: Other image analysis software (e.g., ImageJ) may also be used to manually draw lines for measuring diameters.

35. Calculate the organoid diameter range by subtracting the diameter of the smallest organoid from the diameter of the largest organoid at Day 0. Divide this value by 3 to determine the diameter range for small, medium, and large organoids.

$$Organoid\ diameter\ range = \frac{Largest\ organoid\ diameter - smallest\ organoid\ diameter}{3}$$

36. Assign each organoid into either small, medium, or large groups.
37. Calculate the percentage of each organoid size at each time point by dividing the number of small, medium, or large organoids by the total number of organoids counted per well.

$$Percentage\ of\ (size)\ organoids\ per\ well = \frac{total\ number\ of\ (size)\ organoids\ per\ well}{total\ number\ of\ organoids\ per\ well}$$

a. Example organoid diameters and corresponding sizes are provided in Table 5.
b. In the example provided in Table 5,
   i. Small organoids are <100 μm in diameter.
   ii. Medium organoids are 100 μm–200 μm in diameter.
   iii. Large organoids are >200 μm in diameter.

EXPECTED OUTCOMES

This protocol was used assess the therapeutic efficacy of two STAT3 inhibitors, Ruxolitinib (Figure 2) and Stattic (Figure 3), on gastric tumor organoids derived from *Gp130*^{Y757F} mice. *Gp130*^{Y757F} mice spontaneously develop gastric tumors from 12 weeks of age due to excessive STAT3 activation.[1] Ruxolitinib inhibits activation of Gp130-associated Janus Kinases (JAK) upstream of STAT3 signaling, while Stattic prevents the nuclear translocation of activated STAT3.[12,13]

Organoids were treated with three different concentrations of Ruxolitinib (30nM, 30 μM, 300 μM) or Stattic (1 μM, 3 μM, 10 μM) to produce three outcomes per inhibitor. These include (1) no effect on

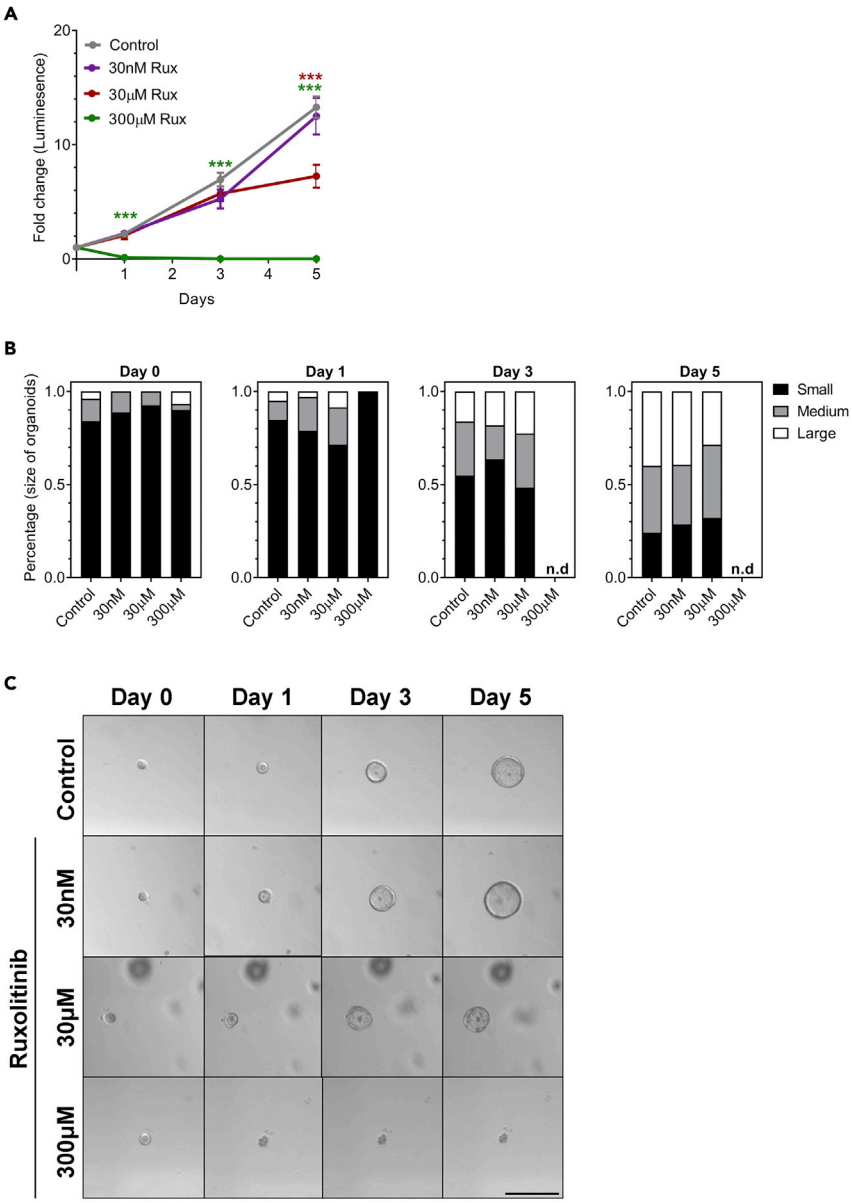

Figure 2. Effect of Ruxolitinib on gastric tumor organoid viability and growth

(A) Viability (luminescence) of *Gp130*^{Y757F} organoids treated with the small molecule inhibitor Ruxolitinib at 30nM (no effect), 30 μM (partial effect), or 300 μM (maximal effect) over a 5-day assay period. Untreated wells were used as a control. *n = 3–6* biological replicates per condition with each biological replicate comprising of technical triplicates.

(B) Percentage of organoid size measured for each condition on the indicated days. n.d., not detected.

(C) Photomicrographs of the same organoid for each condition on the indicated days. Scale bar: 200 μm. Data represent mean ± SEM; ***p < 0.001, with statistical significance determined by two-way ANOVA followed by Tukey's multiple comparison test.

viability (i.e., RLU readout) compared to untreated organoids, (2) partial effect on viability compared to untreated organoids, and (3) maximal effect on viability compared to untreated organoids.

In Figure 2A, the RLUs of untreated organoids increased ~13 fold over a period of 5 days in culture. Treatment of organoids with 30nM Ruxolitinib had no impact on organoid viability compared to the untreated control. However, treatment with 30 μM Ruxolitinib partially reduced viability, and this

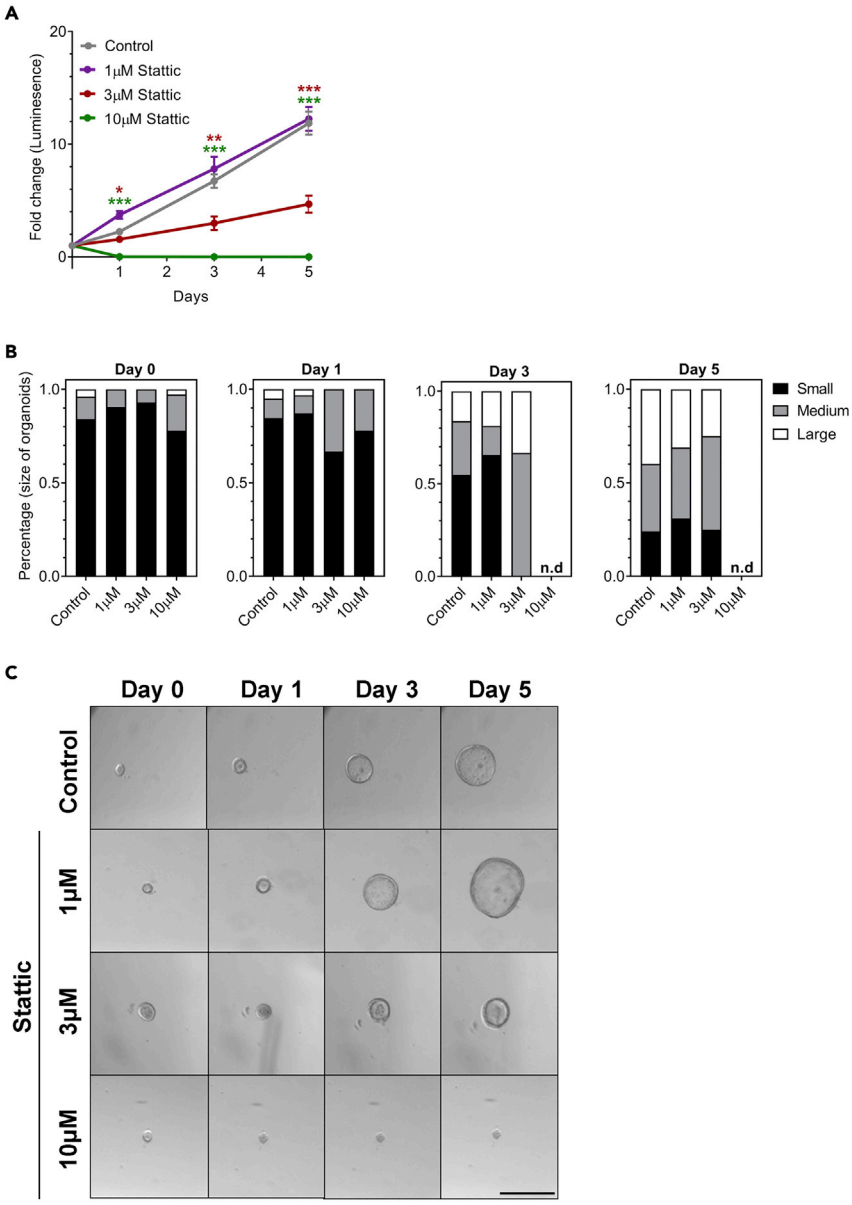

Figure 3. Effect of Stattic on gastric tumor organoid viability and growth

(A) Viability (luminescence) of *Gp130*^{Y757F} organoids treated with the small molecule inhibitor Stattic at 1 μM (no effect), 3 μM (partial effect), or 10 μM (maximal effect) over a 5-day assay period. Untreated wells were used as a control. *n = 3* biological replicates per condition with each biological replicate comprising of technical triplicates.

(B) Percentage of organoid size measured for each condition on the indicated days. n.d., not detected.

(C) Photomicrographs of the same organoid for each condition on the indicated days. Scale bar: 200 μm. Data represent mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, with statistical significance determined by two-way ANOVA followed by Tukey's multiple comparison test.

was further enhanced when Ruxolitinib was used at 300 μM. Similar results were observed following treatment of organoids with increasing doses of Stattic (Figure 3A).

To validate these findings, organoid viability was also assessed by measuring the diameter (a surrogate measure of growth) for the same organoids over the course of treatment. Full-well photomicrographs were obtained using an Olympus IX81 microscope of a single well per condition for each figure

provided after measuring luminescence on Days 0, 1, 3, and 5. The fold-change in diameter of each organoid was quantified over the course of Ruxolitinib or Stattic treatment as a surrogate measure of growth (Figures 2B, 2C, 3B, and 3C). These results are consistent with the luminescence assay, where the lowest dose of each compound had negligible effects on organoid growth. In contrast, 30 μM of Ruxolitinib and 3 μM of Stattic partially inhibited organoid growth, which was further reduced when organoids were treated with either 300 μM of Ruxolitinib or 10 μM of Stattic.

QUANTIFICATION AND STATISTICAL ANALYSIS

A two-way ANOVA followed by Tukey's multiple comparison test was performed using Prism version 9.3.1 (GraphPad). Data represents mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

LIMITATIONS

The RealTime-Glo™ MT Cell Viability Assay measures the metabolic activity of viable organoids, which is provided as a luminescence signal. If the luminescence signal of treated organoids increases at a slower rate and/or stabilizes in comparison to the luminescence signal of untreated organoids, it is difficult to determine whether this is due to organoid senescence or death. For this reason, this protocol combines the luminescence assay with microscopy to longitudinally quantify organoid diameter as a surrogate measure of growth.

TROUBLESHOOTING

Problem 1

Luminescence decreases in control wells prior to assay end-point (Related to step 12). Possible reasons include:

- Organoid density is too high. Over-plating of organoids causes over-crowding and death.
- Toxicity of test compound diluents. Some diluents may interfere with the luciferase reaction and/or may cause premature organoid death if the concentration is too high.

Potential solution

- Optimize the number of organoids plated per well to facilitate robust growth, while also avoiding overgrowth at end-point.
- Treat control organoids with varying concentrations of test compound diluents to determine the optimal concentration that can be used without resulting in toxicity.

Problem 2

High variability in luminescence between technical and biological replicates may be observed if organoids are not consistently plated at the same number per well (Related to step 17).

Potential solution

To ensure even distribution of organoids throughout the *Basement Membrane Matrix* mix, gently resuspend organoids by pipetting the mix after plating 5 wells.

Problem 3

Luminescence does not increase in control wells from baseline measurement (Related to step 12).

Potential solution

The starting number of organoids per well may be too low, which will limit signal intensity. Optimize the density of organoids plated per well to facilitate robust growth, while also avoiding overgrowth at end-point.

Users may also need to wait longer than 16–24 h after plating organoids before obtaining a baseline measurement. If the growth kinetics of selected organoids is slow, allowing additional time for

recovery/establishment will increase the likelihood of acquiring a higher baseline luminescence measurement.

Problem 4

Drug screening results are not reproducible (Related to steps 31–37).

Potential solution

Check the storage conditions and stability of the test compounds, or use fresh aliquots when commencing the assay. Users are advised to avoid freeze-thawing test compounds more than once.

Problem 5

Blurry and/or out-of-focus images of organoids (Related to steps 33–37).

Potential solution

- Recalibrate the microscope to ensure that photos of the entire wells are captured.
- Confirm that the microscope stage position and lower/upper z-stack limits are consistent across the duration of the assay.
- Do not introduce bubbles into the *Basement Membrane Matrix* mix when resuspending and plating organoids.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Ashleigh Poh (ashleigh.poh@onjcri.org.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper did not generate new datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.J.M.; investigation, R.J.M.; writing, R.J.M., M.E., A.R.P.; review & editing, R.J.M., M.E., A.R.P.; supervision, M.E., A.R.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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